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Celebrating David Barrington's Contributions to Fern and Lycophyte Research: Introduction to an American Fern Journal Special Issue

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During the Botany 2019 meeting in Tucson, Arizona, a large group of colleagues convened to celebrate the career achievements of Professor David Stanley Barrington with a colloquium titled "Reticulate evolution and biogeography in ferns and lycophytes – a colloquium honoring Dr. David Barrington." The colloquium was organized by myself, Michael Sundue, and Nikisha Patel, and featured talks by eleven researchers from nine institutions, nearly all of whom are early career researchers. The talks covered a diverse array of topics but were united in their shared focus on themes and approaches advanced by Dave over the course of a career that has spanned six decades.

Dave's career in botany started during his undergraduate studies at Bates College, where he was particularly inspired by Harold E. "Hal" Hackett, a young phycologist who joined the faculty shortly before Dave's freshman year. To complete research for a term paper, Hal suggested that Dave visit the Farlow Herbarium library at Harvard University, where Dave came into contact with his future Ph.D. advisor, Rolla Tryon. Following his graduation from Bates in 1970, Dave started his doctoral studies at Harvard, becoming a member of one of the leading groups of fern systematists of the time. His dissertation research focused on the taxonomy of the tree fern genus *Trichipteris* (Cyatheaceae) (Barrington, 1976; Barrington, 1978); studying this challenging, principally South American group would provide him with a solid foundation that he would build upon later in his career.

In 1974, Dave joined the faculty at the University of Vermont, where he has remained to this day. Shortly after taking this position, Dave shifted his research activities to the genus *Polystichum* (Dryopteridaceae), which has remained a constant focus in his lab since (*e.g.*, Barrington, 1985b; Barrington, 1986; Barrington, 1990; Barrington, 1992; Barrington, 1995; Little and Barrington, 2003; Barrington, 1993; Driscoll and Barrington, 2007; Barrington, 2012; McHenry and Barrington, 2014; Jorgensen and Barrington, 2017; Patel *et al.*, 2018). Key to Dave's long and active career has been his enthusiasm for applying contemporary techniques and approaches in pursuit of the principal goals of his research: resolving reticulate evolutionary histories and understanding plant biogeography. A sabbatical spent at Chris Haufler's lab at the University of Kansas in the spring of 1985 had a particularly transformative impact on Dave's career. The goal of that visit was to learn isozyme electrophoresis, a technique that by that time was widely used in angiosperms but was just gaining traction in the fern and lycophyte community as a powerful way to infer the relationships of taxa in groups with complex

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histories of hybridization and polyploidy. At the time of this sabbatical, the Haufler Lab (then including Tom Ranker, Mike Windham, Charlie Werth, and Paul Wolf) was at the forefront of the isozyme revolution in ferns; research in the Barrington Lab would soon feature this powerful technique (Barrington, Haufler, and Werth, 1989; Barrington, 1990; Barrington, 2003; Driscoll, Gilman, and Barrington, 2003). Later years brought other approaches, including RFLPs (Stein and Barrington, 1990), AFLPs (Koenemann, Maisonpierre, and Barrington, 2011), and Sanger sequencing of both chloroplast (Driscoll and Barrington, 2007; McKeown, Sundue, and Barrington, 2012; McHenry, Sundue, and Barrington, 2013; McHenry and Barrington, 2014; Testo, Watkins, and Barrington, 2015; Morero *et al.*, 2019) and nuclear loci (Dragon and Barrington, 1990; Jorgensen and Barrington, 2017; Lyons, McHenry, and Barrington, 2017; Patel *et al.*, 2018).

With this ever-evolving repertoire of techniques, Dave has successfully led an active research program at the University of Vermont that has contributed significantly to our understanding of plant diversity, especially of ferns of North America, Central America, and East Asia. During his career, Dave has authored over 60 peer-reviewed publications, including 29 in the last ten years.

Even more impressive than the number of publications is the impact that these publications have had in shaping the future of our discipline. Dave's most-cited single-author paper (194 citations at the time of writing, according to Google Scholar) is a review article titled "Ecological and Historical Factors in Fern Biogeography," which was published in the *Journal of Biogeography* nearly thirty years ago (Barrington, 1993). This paper is remarkably successful in its goal of advancing our understanding of fern biogeography through the synthesis of a century of research across diverse disciplines, and examination of citation history shows that it has influenced important advancements in biogeography, in ferns and beyond (DeForest Safford, 1999; Crisp *et al.*, 2001; Kessler, 2001). Even considering the large body of literature it inspired, a close read of Barrington (1993) makes it clear that many of the important questions raised in that work (How common is hybridization in tropical ferns? How closely do montane habitat islands mirror patterns observed in oceanic island biogeography?) remain to be fully addressed, providing fertile ground for future generations of biogeographers.

Two other papers, each published in the *American Fern Journal* and with Dave as the lead author, stand out to me as being particularly influential contributions to the study of reticulate evolution in ferns and lycophytes. The first of these, "Systematic inferences from spore and stomate size in the ferns" by Barrington, Paris, and Ranker (1986) established that closely related ferns of different ploidy levels could, in most cases, be reliably distinguished by the size of their stomata and spores. This fast, low-cost, and effective approach has been a key part of the fern systematist's toolkit ever since, and has played an important role in resolving previously intractable polyploid complexes in genera like *Myriopteris* (Grusz, Windham, and Pryer, 2009), *Cystopteris* (Haufler and Windham, 1991; Rothfels *et al.*, 2014), *Polypodium* (Haufler and Windham, 1991), and *Astrolepis* (Benham, 1992; Beck *et al.*, 2010). Driven by an insatiable intellectual curiosity and a desire to see a good method become better, Dave, along with students Nikisha Patel and Morgan Southgate, recently revisited this topic, providing even more sophisticated insights into the relationship between spore size, ploidy level, and genome size in ferns (Barrington *et al.*, 2020). The second, "Hybridization, reticulation, and species concepts in the ferns" by Barrington, Haufler, and Werth (1989), carefully examines how hybridization and polyploidy complicate the application of species concepts in ferns and provides thoughtful suggestions for how fern and lycophyte biologists can make sense of extant fern diversity (namely by recognizing evolutionary cohesive lineages of hybrid origin as species, in most cases). With its cogent responses to fundamental questions of evolutionary biology ("Are there two distinguishable kinds of species, hybrid species (nothospecies) and divergent species (orthospecies), as Wagner contends (1969, 1983)?"), this paper strikes me as even more important today than when I first read it as an undergraduate student, nearly a decade ago.

In addition to maintaining a productive research career, Dave has been an exceptional mentor to generations of students at the University of Vermont. He has advised 21 Master's and Ph.D. students and inspired hundreds of undergraduate students, especially participants in his tropical botany field course, which he has taught in Costa Rica every other year since 1979. For these exceptional efforts in teaching, he has received two awards at the University of Vermont: the Joseph E. Carrigan Award for Excellence in Undergraduate Teaching and the Kroepsch-Maurice Outstanding Teacher Award. As a former student of Dave's who had the good fortune to take several of his courses during graduate school, I strive to emulate his enthusiasm and success in inspiring students in my own classes.

As I reflect on Dave's successful career, I would be remiss if I failed to acknowledge the important role of his longtime collaborator and partner of more than 35 years, Cathy Paris. In addition to co-authoring several papers with Dave (Barrington, Paris, and Ranker, 1986; Barrington and Paris, 2007), Cathy has made several other important contributions to fern biology, especially on systematics of the maidenhair ferns (*Adiantum*) (Paris and Windham, 1988; Paris, 1991). As he has attested to in the acknowledgments of dozens of publications, Dave has benefitted tremendously from scientific conversations and editorial advice from Cathy, who is both an accomplished systematist and talented editor. Put simply, it would be hard to overstate the extent to which Cathy has helped make Dave the exceptional botanist, teacher, and mentor that he is.

Circling back to the colloquium mentioned in the beginning of this piece, a glance at that event's lineup of speakers highlights Dave's success in inspiring future generations of fern and lycophyte biologists: seven of the talks were presented by current or former Barrington Lab members, and all the talks focused on themes advanced by Dave during his career. Following an introduction by the colloquium organizers, the session included eleven talks, which are listed below:

1. "David Barrington: fern fanatic, consummate collector, creative collaborator, and *Polystichum* whisperer par excellence" -Christopher Haufler
2. Persistence pays off: long term field work in the Rockies yields another species of reticulate origin in the Moonwort Complex" -Arthur Gilman, Steve Popovich, and Don Farrar
3. "Ecological outcome of allopolyploidy in *Adiantum* (Pteridaceae): niche intermediacy and expansion into novel habitats" -Morgan Southgate, David Barrington, and Nikisha Patel
4. "Long-distance dispersal and geographical range expansions of the polyploid lineages in *Deparia* ferns (Athyriaceae; Polypodiales)" -Li-Yaung Kuo
5. "Species boundaries and population structure in the pan-tropical fern genus *Ceratopteris* (Pteridaceae)" -Sylvia Kinosian and Paul Wolf
6. "Ten years later: revisiting the *Myriopteris yavapensis* complex (Pteridaceae) in the genomics era" -Amanda Grusz
7. "600-species phylogenomic dataset provides insights into historical global biogeography of the Thelypteridaceae, with special reference to Hawaiian and African taxa" – Susan Fawcett, David Barrington, Michael Sundue, Gordon Burleigh, Emily Sessa, GoFlag consortium, Li Yaung Kuo, and Alan Smith
8. "Global patterns of fern diversification" -Jacob Suissa
9. "Polyploid speciation in the *Polystichum* of the Sierra Talamanca" -Stacy Jorgensen
10. "Reticulate evolutionary histories of the apomictic lineages in East Asian *Polystichum*" -Nikisha Patel
11. "Fifty years of fern hybridization and historical biogeography: new perceptions and new tools, all addressing the same fundamental questions" -David Barrington

This special issue of the American Fern Journal builds on this colloquium and includes six papers that explore diverse topics ranging from resolving reticulate complexes to Neotropical fern biogeography. The series begins with two studies focused on polyploid complexes. In the first, Jorgensen and Barrington (151–164) use nuclear DNA sequence data to provide new perspectives on the evolution of Central American *Polystichum*, a group that has been a main focus of Dave's research for more than 35 years. In the second, Popovich, Farrar, and Gilman (165–182) describe a new allotetraploid *Botrychium* from western North America based on combined datasets of allozymes and detailed morphological measurements. Following these, Fawcett (183–192) describes a new species of *Goniopteris* (Thelypteridaceae) known only from a single location in southwestern Dominican Republic. The fourth contribution is by Kinosian, Pearse, and Wolf (193–210), who use a RADseq approach to highlight widespread hybridization in *Ceratopteris* (Pteridaceae) and possible undescribed lineages. The fifth paper in the issue by Suissa and Sundue (211–232) uses a large specimen-based dataset and a phylogenetic framework to revisit classic hypotheses of Neotropical fern biogeography proposed by Rolla Tryon nearly a half-century ago (Tryon, 1972). In the issue's final contribution, Barrington (233–254) presents a review of biogeography of polyploid ferns, focusing on six groups of ferns that he and his students studied extensively over the last four decades.

Together, the articles in this issue highlight the extent to which David Barrington's contributions have influenced a generation of innovative and active fern and lycophyte biologists and helped usher in a new and exciting era of discovery. I hope that readers of this special issue will enjoy reading these articles as much as I have enjoyed working with them through the editorial process.

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Speciation and Reticulation in the *Polystichum* Allotetraploids of the Costa Rican Cordillera Talamanca

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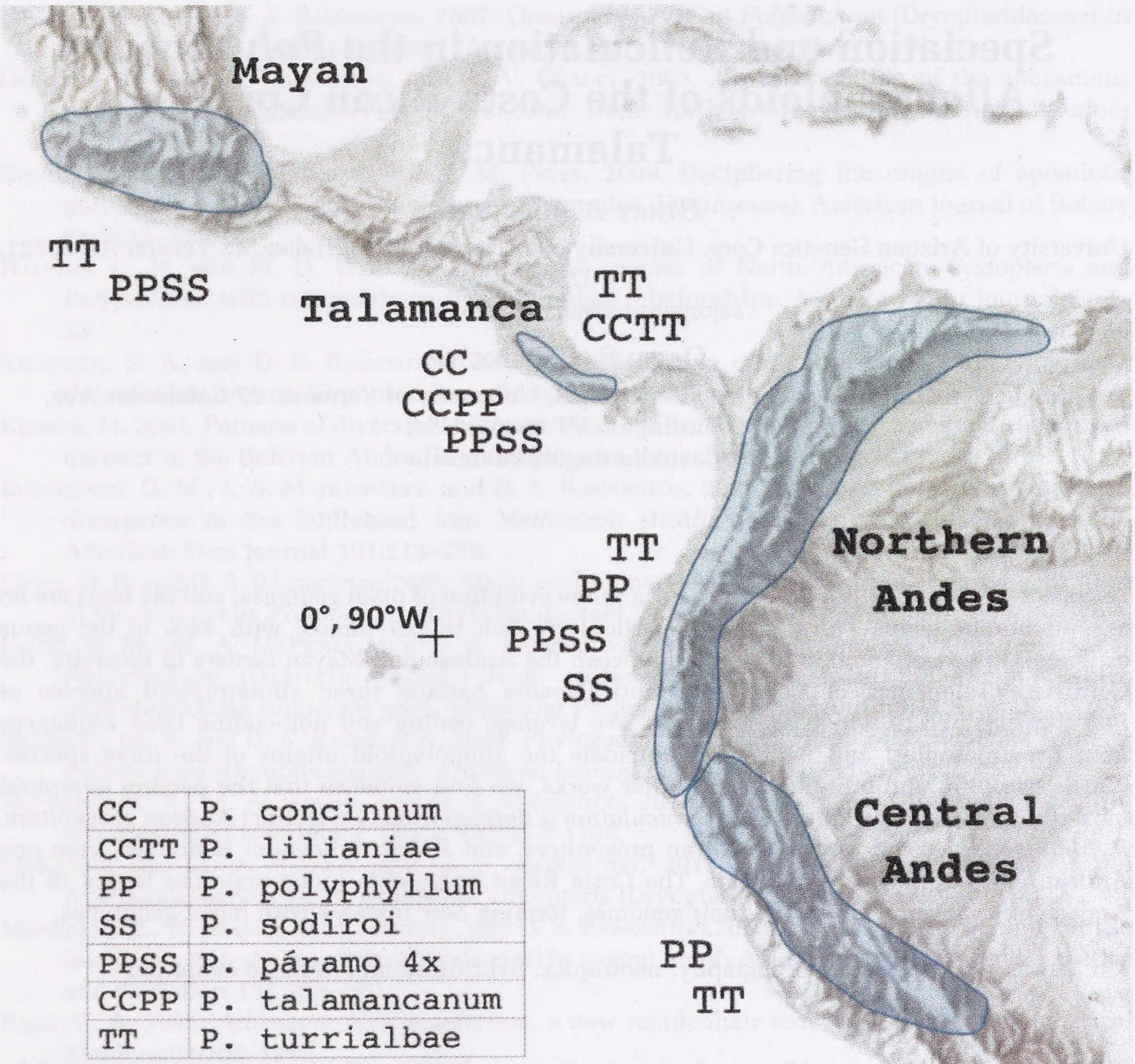
Abstract.—Polyploidy is a prominent force in the evolution of plant genomes, and the ferns are no exception. The genus *Polystichum* is particularly rich in polyploids, with 44% of the genus estimated to be polyploid. A waypoint between the Andean and Mayan centers of diversity, the Cordillera Talamanca of Costa Rica and Panama harbors three allotetraploid species of *Polystichum*, two of which are endemic. We leverage coding and non-coding DNA sequences from the chloroplast and nucleus to elucidate the allopolyploid origins of the three species. Consistent with, and building upon earlier works, we find evidence that the páramo tetraploid most recently named as *Polystichum orbiculatum* is derived from two distinct Andean progenitors, *P. lilianiae* is derived from two Mayan progenitors, and *P. talamancanum* is derived from one Andean and one Mayan progenitor. The Costa Rican polyploids incorporate the legacy of the Andean and Mayan regions into their genomes, forming new lineages with novel genotypes.

Key Words.—Polyploidy, biogeography, neotropics, hybridization, reticulate evolution

Polyploidy is a significant factor in the evolution of plant genomes, notably so in the ferns. Approximately one-third of fern and lycophyte species are estimated to be polyploid (Wood *et al.*, 2009). The genomes of allopolyploids combine genetically distinct progenitors, and in the ferns often originate via hybridization and subsequent chromosome doubling. The combination of the genetic signatures of otherwise reproductively isolated progenitor lineages bestows novel genotypes on the nascent polyploid species, as evidenced by the fixed heterozygosity frequently observed in allopolyploids (Soltis *et al.*, 2000). Polyploid speciation has been investigated in the ferns using morphology (Wagner, 1954), chromosome number and pairing behavior (Manton, 1950), isozyme variation (Barrington, 1990; Haufler, 1987), and DNA sequencing (Grusz, Windham, and Pryer, 2009; Sigel, Windham, and Pryer, 2014).

The nearly cosmopolitan genus *Polystichum* Roth (Dryopteridaceae) comprises at least 400 species (Barrington, unpublished). Approximately 44% of species with chromosome counts are polyploid (Lóve, Lóve, and Pichi-Sermolli, 1977; Manton, 1950). *Polystichum* is largely a tropical to warm-temperate genus, with many species occurring in wet montane forests and high-elevation shrubby grasslands (referred to as páramos in the neotropics),



CC	P. concinnum
CCTT	P. lilianiae
PP	P. polyphyllum
SS	P. sodiroi
PPSS	P. páramo 4x
CCPP	P. talamancanum
TT	P. turrialbae

FIG. 1. Map of the study region and geographical distribution of the three tetraploids and their hypothesized progenitors. Blue shading highlights the Mayan and Andean centers of diversity as well as the Cordillera Talamanca of Costa Rica and Pánama.

most diverse in eastern Asia and tropical America. Three major centers of diversity are found in the neotropics (Fig. 1), with 29 species in the central and northern Andes (Kessler, Smith, and Sundue, 2005; McHenry and Barrington, 2014), 18 species in Mexico and Guatemala (Mickel and Smith, 2004; Stolze, 1981), and 31 species in the Greater Antilles (Mickel, 1997). Neotropical *Polystichum* species form a monophyletic group, within which are clades comprising species from Mexico and Guatemala (which we refer to as the Mayan lineage) and the Andean exindusiate lineage found in the northern and central Andes (Driscoll and Barrington, 2007; McHenry and Barrington, 2014). A waypoint between the Andean and Mayan diversity hotspots, the highlands of Costa Rica and western Panamá (hereafter the Cordillera Talamanca)

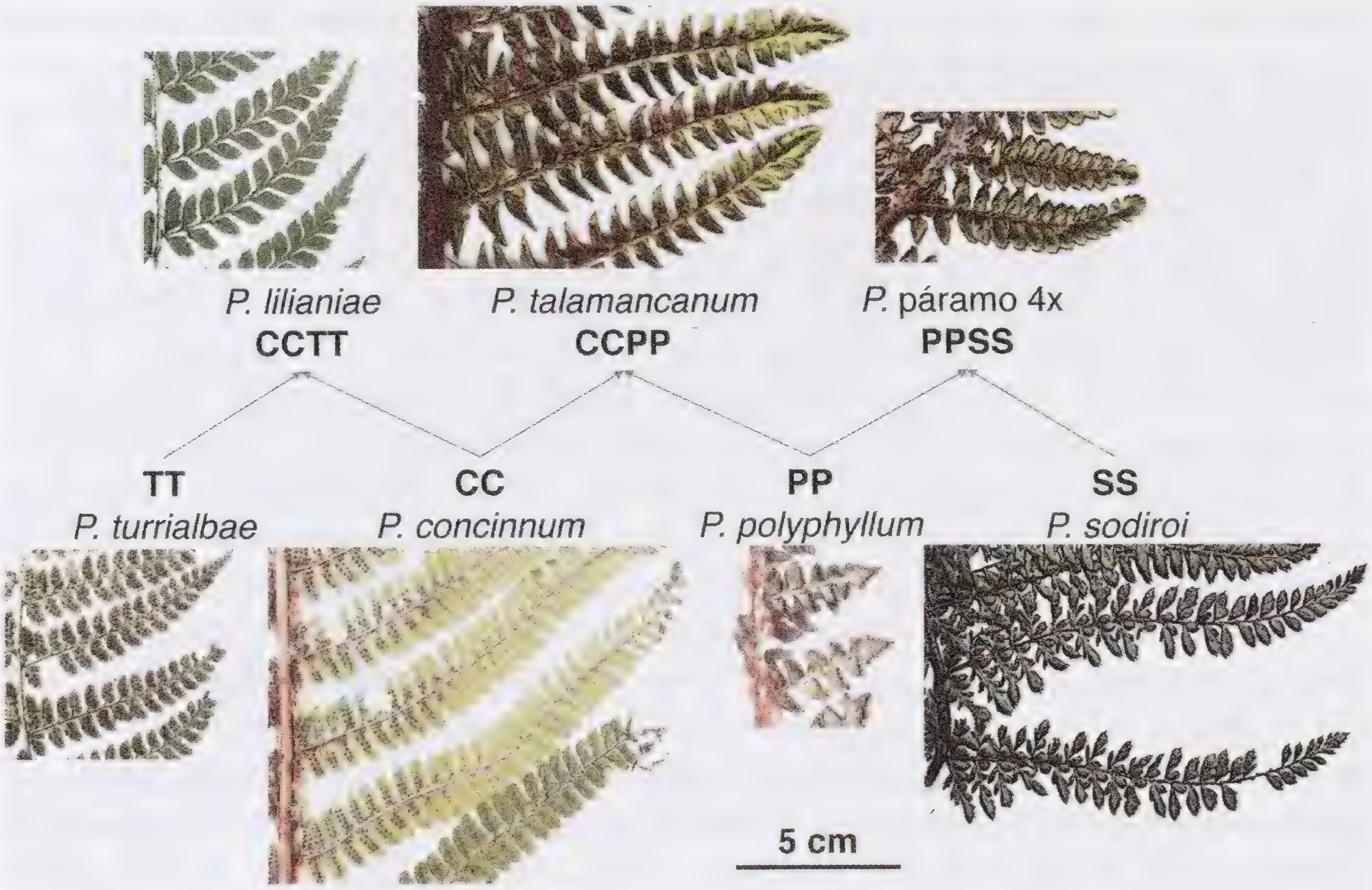


FIG. 2. Reticulogram depicting the relationships of the three tetraploids (top row) and their putative diploid progenitors (bottom row), with photographs of representative pinnae. Specimens: *P. lilianiae*, S. A. Jorgensen 903; *P. talamancanum*, J. Kluge 1428; *P.* páramo tetraploid, D. Barrington 2143; *P. turrialbae*, D. Little 295; *P. concinnum*, D. Barrington 2292; *P. polyphyllum*, H. Whidden 104; *P. sodiroi*, M. Sundue 1060. All collections held at VT. Photographs: D. Barrington.

combine the floristic influence of the Mayan region to the north and the Andean region to the south. In general, the species occurring in the páramos of the Cordillera Talamanca belong to the Andean lineage, while those found in the forests belong to the Mayan lineage (Barrington, 2005). The Cordillera harbors three allotetraploid species, two of which are endemic to the region, along with nine diploids and an array of hybrids (Barrington, 2011).

Of the three Costa Rican tetraploids, *Polystichum lilianiae* Barrington occurs at the lowest elevation, in montane rain forests between 2500 and 3000 m (Barrington, 2011). It is endemic to the Cordillera Talamanca. This species is characterized by its narrow rhizomes supported by adventitious roots; relatively small, twice-pinnate fronds broadest at the base; stramineous petiole scales with sharply defined fuscous centers; and indusia ca. 1.0 mm (Fig. 2). It is tetraploid with a meiotic count of n=82, and exhibits fixed heterozygosity at an array of isozyme loci. The heterozygous isozymes display a summation of the isozyme profile of the diploid *Polystichum turrialbae* Christ and a second unknown Mayan progenitor (Barrington, 2003). This species has not been previously subjected to genetic study based on DNA sequencing techniques.

The allotetraploid *Polystichum talamancanum* Barrington is also endemic to the Cordillera Talamanca, where it is commonly found at 3100 to 3400 m in páramos, shrubby dwarf forest, and disturbed sites in montane rain forest

(Barrington, 2011). It is a stiffly erect plant, twice-pinnate with ascending pinnae; pointed, revolute pinnules with strong apical spines; dark, long-ciliate petiole scales, and—rarely—partially developed indusia (Fig. 2). A shade form of the plant with flat pinnules also occurs. Based on chloroplast DNA markers, it is phylogenetically allied with the Mayan lineage (McHenry and Barrington, 2014). *Polystichum talamancanum* is tetraploid with a meiotic count of n=82. Cytologic and isozyme evidence demonstrated that it is an allotetraploid derived from diploid *Polystichum concinnum* Lellinger ex Barrington and a second unknown progenitor (Barrington, 1990). The genome of *P. talamancanum* has been denoted CCXX, indicating the contributions of *P. concinnum* (C) and the unknown progenitor (X) (Barrington, 1990). The occasional presence of partially developed indusia suggests that *P. talamancanum* is the result of a cross between Mayan and Andean progenitors (Barrington, 1985).

The third allotetraploid found in Costa Rica is currently undescribed. It has been previously included under the name *Polystichum orbiculatum* (Desvaux) Fée & Remy in Gay. There are considerable taxonomic issues with this name, as it currently includes diploid and multiple tetraploid cytotypes based on spore size data (Barrington, unpublished) and genetic data from the *pgiC* gene (Lyons, McHenry, and Barrington, 2017). Here, we refer to the third allotetraploid of the Cordillera Talamanca as the *Polystichum* páramo tetraploid. This species is found in high páramos from northern Central America south through the Northern Andes; it is sometimes confused with *P. talamancanum* in Costa Rica. Found at 3400 to 3800 m, it occurs most frequently in páramos. In exposed conditions at high elevation it is erect, dwarfed, twice-pinnate, with short, strongly ascending pinnae, strongly revolute pinnules with little spinule development, and amber to stramineous petiole scales; indusia are always absent (Fig. 2). When found in shade it has a more relaxed leaf architecture with flat, rounded pinnules. Based on a chloroplast DNA phylogeny, it is allied with the Andean lineage (McHenry and Barrington, 2014). The páramo tetraploid has a meiotic count of n=82 (Barrington, 1990). The cytology and isozymes show that it is allotetraploid, with one of its progenitors shared with *P. talamancanum* (Barrington, 1990). The genome of the páramo tetraploid has been denoted XXYY, as it combines the unknown progenitor of *P. talamancanum* (X) with a second unknown progenitor (Y) (Barrington, 1990). Based on available morphological and genetic evidence, it is very likely that the progenitors of the páramo tetraploid are both of the Andean lineage.

Here, we explore the origin and evolutionary history of the three tetraploid *Polystichum* species we have encountered on the Cerro de la Muerte of the Costa Rican Cordillera Talamanca. In order to further evaluate and build upon our existing understanding, we introduce direct and cloned sequences from the nucleus, which, along with existing chloroplast sequence data, we use to characterize the component subgenomes of the allotetraploids. We interpret these data in a phylogenetic context, drawing on three data sets to propose the identity and relationships of the missing progenitors.

MATERIALS AND METHODS

Taxonomic sampling.—For this study, 17 species were included (Appendix 1). The circumboreal species *Polystichum lonchitis* (L.) Roth was chosen as an outgroup, as it has been shown to be sister to all neotropical *Polystichum* in earlier work (Driscoll and Barrington, 2007; Little and Barrington, 2003). The ingroup comprised the 12 species that are known to occur in Costa Rica, including nine diploids and three polyploids (Barrington, 2011), along with geographically and morphologically appropriate candidate diploid progenitors. Two individuals for each tetraploid were included.

DNA isolation, amplification, and sequencing.—DNA bench technique and analysis methods followed closely the methods detailed in Jorgensen and Barrington (2017). Genomic DNA was extracted using a technique based on the CTAB method (Dempster *et al.*, 1999; Doyle and Doyle, 1987). Fresh or dried leaf tissue was ground in extraction buffer (1.4 M NaCl, 4% PVP, 2% CTAB, 100 mM Tris-HCl, 20 mM EDTA, 0.25% beta-mercaptoethanol), incubated at 55 °C for 1–24 hr, extracted with 24:1 chloroform:isoamyl alcohol, and precipitated in 0.08 volumes of potassium acetate and 0.54 volumes of isopropanol. The precipitate was pelleted by centrifugation; the pellets were washed with 70% and 95% ethanol, dried, and resuspended in sterile water.

CHLOROPLAST MARKERS.—Four chloroplast markers were used: the *rbcL* gene (Little and Barrington, 2003); the *trnL-trnF* intergenic spacer (*trnLF* primers e and f, Taberlet *et al.,* 1991); and the *rps4* gene sequenced together with the *rps4-trnS* intergenic spacer (*rps4-trnS*, Souza-Chies *et al.*, 1997). PCR products were cleaned with ExoSAP-IT (Applied Biosystems) and sequenced using the ABI Prism BigDye Terminator cycle sequencing kit (Applied Biosystems) on an ABI Prism 3130x1 sequencer (Vermont Integrative Genomics Resource DNA Facility, Burlington, Vermont).

NUCLEAR MARKERS.—Two nuclear markers, *gapCp* spanning exons 8–10 and *pgiC* spanning exons 14–16, were amplified as above using published primer sets (Ishikawa, Ito, and Kurita, 2002; Schuettpelz *et al.*, 2008). The resulting amplicons were resolved by gel electrophoresis, then excised and purified with the PrepEase extraction kit (Affymetrix). Products from diploids were sequenced directly. Products from the tetraploids *Polystichum lilianiae,* the páramo tetraploid, and *P. talamancanum* were cloned with the TOPO TA cloning kit (Invitrogen). In order to capture both homoeologues, the cloned products were amplified from the purified plasmids of 6–10 colonies. Sequencing was performed as described above.

Alignment and phylogenetic analysis.—Chromatograms were inspected visually, assembled into contigs using Geneious 10.2.6 (http://www.geneious.com/), and aligned with MAFFT 7.45 (Katoh *et al.*, 2002; Katoh and Standley, 2013). Indels were scored using SeqState 1.4.1 (Müller, 2005) using Simmons and Ochoterena simple indel coding (Simmons and Ochoterena, 2000). Concatenation was performed using Sequence Matrix 1.8 (Vaidya, Lohman, and Meier, 2011).

Maximum parsimony (MP) analyses were completed using TNT 1.5 (Goloboff and Catalano, 2016; made available by the Willi Hennig Society). Characters were unordered and equally weighted. A heuristic search was executed with 1,000 ratchet replicates, 200 ratchet iterations with up-and-down weights set at 5%, and 20 trees retained per ratchet, followed by tree-bisection-reconnection branch swapping. Bootstrapping was performed with 1,000 replicates of 10 ratchets, with 20 trees retained per ratchet, saving only the strict consensus tree (Felsenstein, 1985).

Bayesian inference was completed with MrBayes 3.2.6 (Huelsenbeck and Ronquist, 2001; Ronquist *et al.*, 2012) using a GTR-gamma substitution model. Markov Chain Monte Carlo analyses were executed using four heated chains, with 1,000,000 generations sampling every 200 generations, and a 10% burn-in phase. A 50% majority-rule consensus was then calculated.

Results

Chloroplast phylogeny.—Aligned *rbcL* sequences were 1010 bp in length, 11 (1.1%) of which were phylogenetically informative. Aligned *rps4* sequences were 808 bp in length, 24 (3.0%) of which were phylogenetically informative. The aligned *trnLF* sequences were 380 bp in length, 6 (1.6%) of which were phylogenetically informative. Parsimony and Bayesian analyses of the concatenated data set retrieved topologically congruent consensus trees (Fig. 3).

The neotropical species comprising the ingroup resolve into a clade of species from the Andean lineage (1.0 PP, 100% BS) and a clade of species from the Mayan lineage (1.0 PP, 100% BS) as in all previous studies. *Polystichum speciosissimum* (Kunze) R.M.Tryon & A.F.Tryon, disjunct between Mexico and Costa Rica, is sister to the rest of the neotropical species we sampled. Within the Mayan clade, *P. lilianiae* and *P. talamancanum* resolve in a clade with *P. concinnum* with full support (Fig. 1: 1.0 PP, 100% BS). A second accession of *P. lilianiae* is sister to a poorly resolved group comprising *Polystichum hartwegii* (Klotzsch) Hieron., *Polystichum muricatum* (L.) Fée, and *P. turrialbae*. Within the Andean clade, we retrieved two groups: one of predominantly páramo species (1.0 PP, 100% BS) and one of predominantly forest species (1.0 PP, 100% BS). The páramo tetraploid resolves in the páramo clade, sister to *Polystichum polyphyllum* (C.Presl) C. Presl (0.64 PP).

Nuclear phylogeny.—Aligned *gapCp* sequences were 545 bp in length, of which 32 (5.9%) were phylogenetically informative. Parsimony and Bayesian analyses of the *gapCp* data set retrieved topologically congruent consensus trees (Fig. 4). The *gapCp* phylogeny robustly resolves the Andean (0.94 PP, 100% BS) and Mayan (1.0 PP, 100% BS) clades (Fig. 4). Two *gapCp* variants were recovered for each tetraploid. *Polystichum lilianiae* yielded a variant (T) that resolves sister to *P. turrialbae* (0.95 PP, 100% BS). *Polystichum lilianiae* and *P. talamancanum* both yielded a variant (C) which is at an unresolved position in the Mayan clade along with *P. concinnum*, *P. talamancanum*, and *P. hartwegii* (0.92 PP, 100% BS). *Polystichum talamancanum* and the páramo

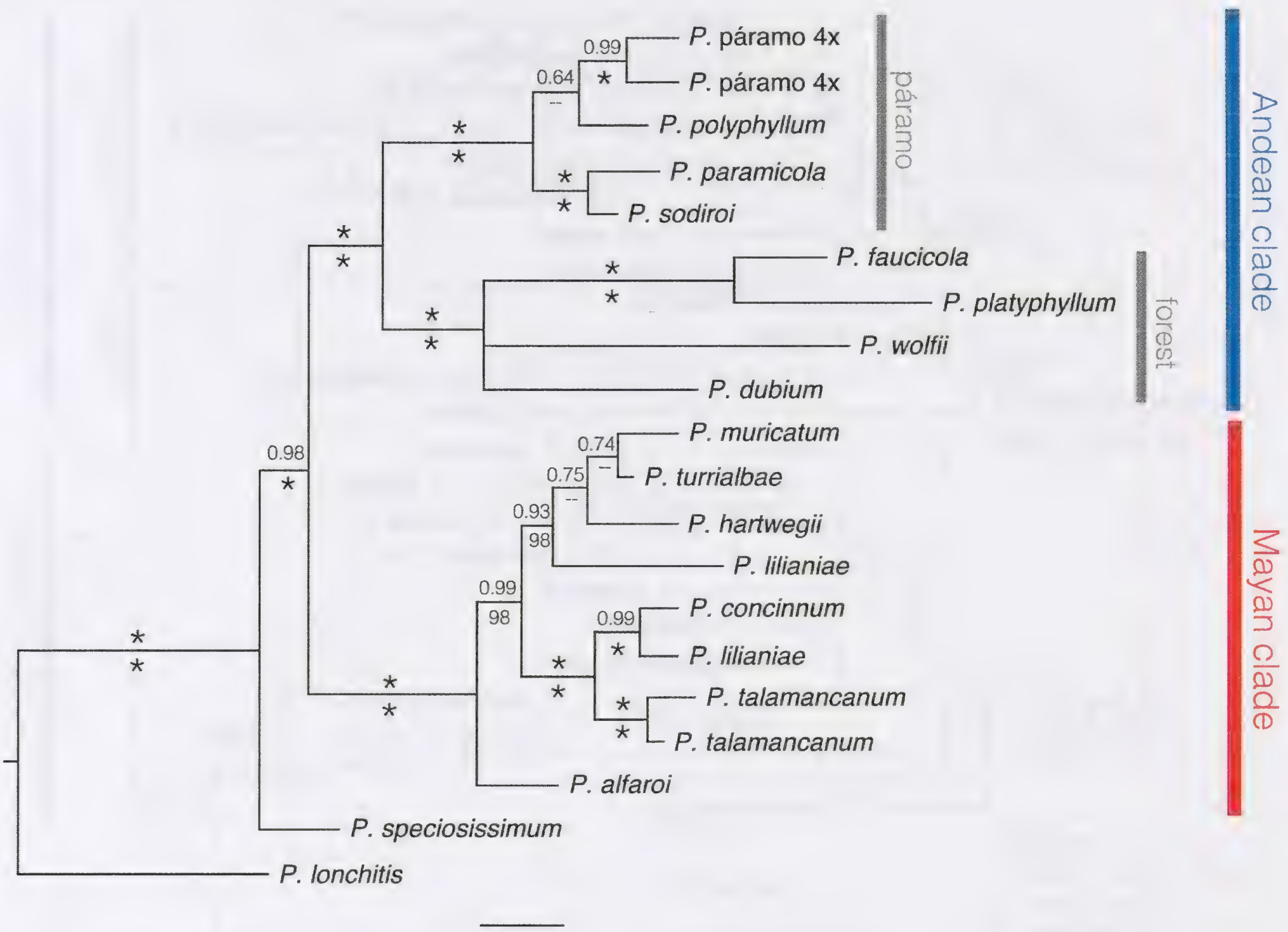


FIG. 3. Fifty-per cent majority rule phylogram inferred from combined plastid markers *rbcL*, *rps4-trnS*, and *trnLF* using Bayesian Inference. Posterior probability (PP) given above nodes; parsimony bootstrap (BS) support given below. Asterisk denotes 1.0 PP or 100% BS support. Nodes collapsed where support is below 0.60 posterior probability.

tetraploid each yielded a variant that resolves in the Andean páramo lineage with diploid *P. polyphyllum* (0.99 PP, 63% BS); we call this variant P. And finally, the páramo tetraploid yielded a second variant that falls in a poorly resolved area of the páramo clade near *P. sodiroi* and diploid *Polystichum paramicola* Kessler and A.R. Sm. (0.98 PP, 98% BS); we call this variant S.

Aligned *pgiC* sequences were 591 bp in length, 13 (2.1%) of which were phylogenetically informative. However, the resolving power of the variable characters was minimal, and the resulting phylogeny was primarily a polytomy for the ingroup taxa (data not shown). Although the *pgiC* nucleotide variation does not help to resolve phylogenetic relationships, several indels found in intron 15 witness the contribution of the sampled diploids to the three polyploid species (Fig. 5). Three indels are important here, numbered from the beginning of *pgiC* intron 15: At nucleotide position 94, a 3 three base-pair insertion is shared by *P. turrialbae* and *P. lilianiae* variant T. At position 294, a one base-pair deletion is shared by *P. concinnum*, *P. lilianiae* variant C, and *P. talamancanum* variant C. At position 228, a ten base-pair deletion is shared by *P. sodiroi* and the páramo tetraploid variant S.

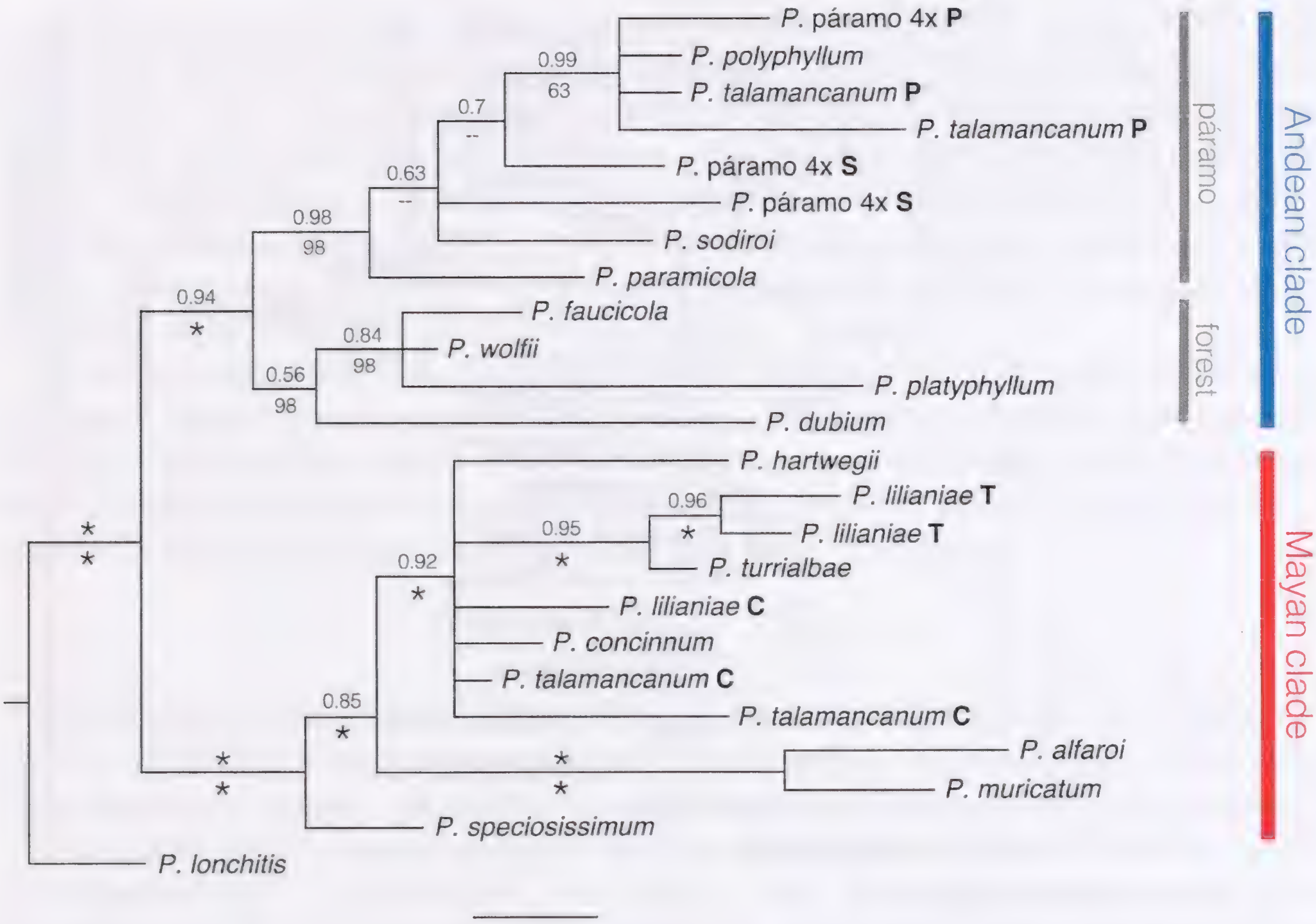


FIG. 4. Fifty-per cent majority-rule phylogram inferred from nuclear markers *gapCp* using Bayesian Inference. Posterior probability (PP) given above nodes; parsimony bootstrap (BS) support given below. Asterisk denotes 1.0 PP or 100% BS support. Nodes collapsed where support is below 0.60 posterior probability.

DISCUSSION

Origin of Polystichum lilianiae.—The two genetic variants recovered from *Polystichum lilianiae* share affinities with Mayan diploids *P. concinnum* and *P. turrialbae*, thus we denote the tetraploid genome of *P. lilianiae* CCTT. The *P. turrialbae* contribution (T) is clearly supported in the *gapCp* phylogeny (Fig. 4, 0.95 PP, 100% BS). Additionally, an insertion in *pgiC* intron 15 is synapomorphic in *P. turrialbae* and *P. lilianiae* variant T (Fig. 5). These results are consistent with the earlier work done with isozymes (Barrington, 2003). The *P. concinnum* variant (C) is robustly supported as a maternal progenitor in the chloroplast phylogeny (Fig. 3, 1.0 PP, 100% BS). Additionally, there is a shared deletion in *pgiC* intron 15 that is synapomorphic in *P. concinnum* and *P. lilianiae* variant C (Fig. 5). That the two *P. lilianiae* accessions resolve at different positions in the chloroplast phylogeny suggests a minimum of two origins (Fig. 3).

In his treatment of the Costa Rican *Polystichum* species, Barrington (2011) predicted that the unidentified progenitor of *P. lilianiae* would have dark petiole scales and small indusia (≤0.6mm), with the basal pinnae the broadest and oblique pinnule attachment, and is likely of the Mayan lineage. The current genetic data suggest *P. concinnum* is the second progenitor of *P.*

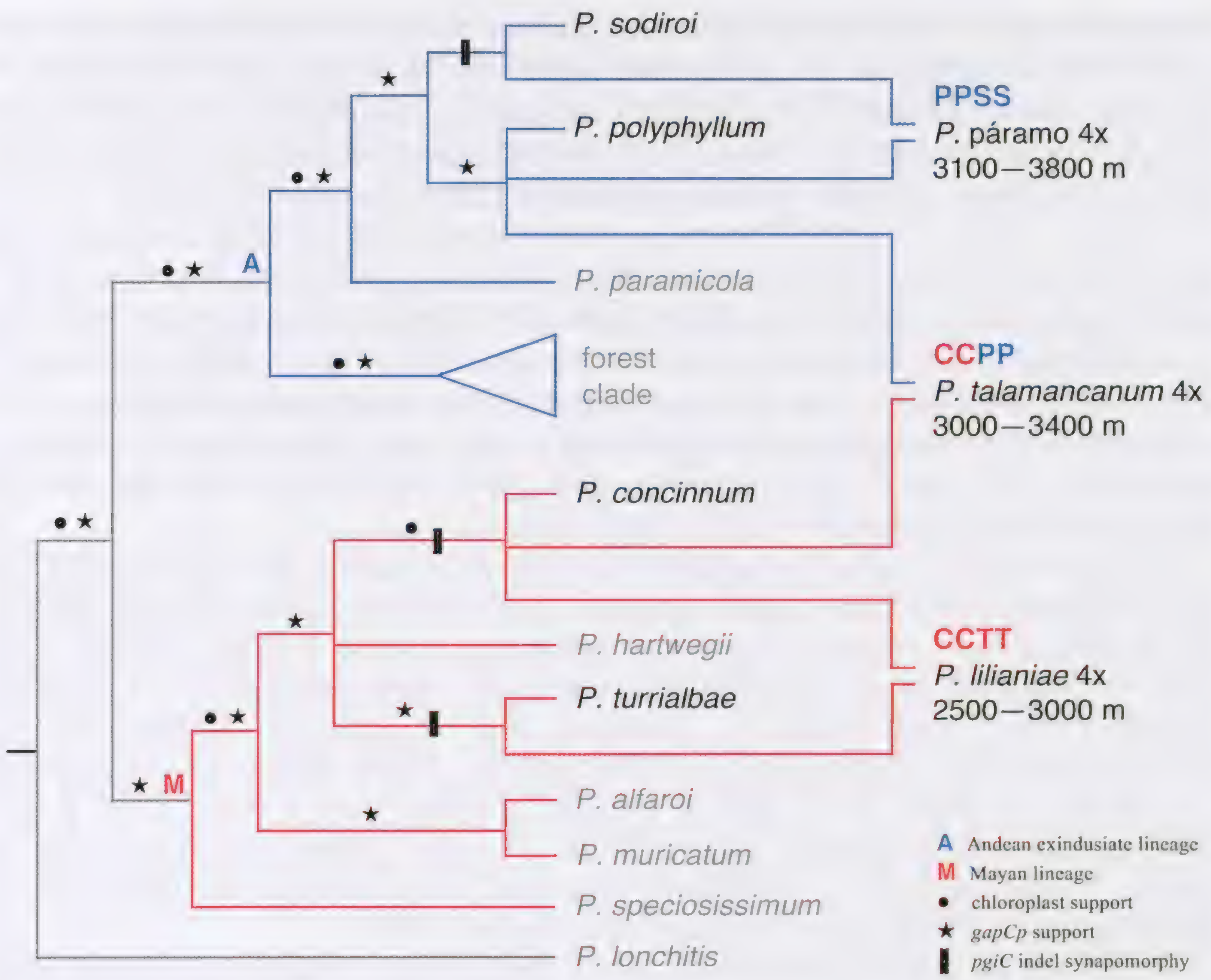


FIG. 5. Cladogram showing the relationships of the three tetraploids of the Cordillera Talamanca and their progenitors. Andean and Mayan lineages marked with A and M, respectively. Branches supported by the chloroplast data marked with a circle; branches supported by the *gapCp* data marked with a star; synapomorphic indels in *pgiC* intron 15 shown with bold tick marks.

lilianiae. How does this compare with the morphology? *Polystichum concinnum* indeed has dark petiole scales and small indusia, but is broadest above the base of the lamina and pinnules are attached at right angles. *Polystichum lilianiae* is intermediate between *P. concinnum* and *P. turrialbae* for petiole scale color, the ratio of lamina length:width, basal pinnule length, and indusium size. It is like *P. turrialbae* in its narrow rhizome diameter, petiole scale size, and the ratio of pinna length:width; it resembles *P. concinnum* in pinna shape. *Polystichum lilianiae* is transgressive relative to the currently proposed progenitors for lamina size, lamina shape, and pinnule attachment. Although transgressive for several morphological characters, *P. lilianiae* is intermediate or like one parent for a substantial set of characters.

Origin of Polystichum talamancanum.—Considering the two genetic variants recovered from *Polystichum talamancanum*, one shares affinities with Mayan diploid *P. concinnum* (variant C) and one with the Andean páramo diploid *P. polyphyllum* (variant P). Variant P resolves in the *gapCp* tree in a clade with *P. polyphyllum* and the páramo tetraploid variant P (Figure 4: 0.99 PP, 63% BS). Thus, the shared progenitor and source of variant P in the

P. talamancanum and the páramo tetraploid genomes is *P. polyphyllum*. Poor resolution observed in the *gapCp* tree presents us with a polytomy with *P. talamancanum* variant C, *P. lilianiae* variant C, and diploids *P. concinnum* and *P. hartwegii* (0.92 PP, 100% BS). However, an indel from *pgiC* intron 15 is shared by the C variant of both tetraploids and *P concinnum*, but not *P. hartwegii* (Fig. 5). Additionally, the close relationship between *P. talamancanum* and *P. concinnum* is strongly supported in the chloroplast tree (Fig. 1, 1.0 PP, 100% BS). These lines of evidence, taken together with the foundational work in cytology and isozymes (Barrington, 1990), provide a convincing argument that *P. concinnum* is the maternal progenitor of *P. talamancanum*. Building on earlier work, we have identified the missing progenitor "X" as *P. polyphyllum*, and now are able to denote the *P. talamancanum* genome as CCPP (Fig. 2; Fig. 5).

The genome of *Polystichum talamancanum* is unique in that it integrates the genetic features of a Costa Rican endemic forest species allied with the Mayan clade with a high páramo Andean exindusiate, to form a novel lineage. This species is found at an intermediate elevation range relative to its entirely Mayan-derived counterpart *P. lilianiae* and its entirely Andean-derived counterpart, the páramo tetraploid (Barrington, 2011). When found growing in full sun, it looks like a typical páramo species, with a stiffly erect habit, strongly ascending pinnae, and revolute pinnules. When found growing in shade, it takes on traits of its forest progenitor. Its occasional vestigial indusia are a hallmark of its mixed heritage (Barrington, 1985).

Origin of the páramo tetraploid.—The two genetic variants recovered from the páramo tetraploid both fall in the Andean páramo clade, and yet are distinct from one another, consistent with earlier work (Barrington, 1990; McHenry and Barrington, 2014). Variant P, as described above, is shared with *P. talamancanum* and resolves with diploid *P. polyphyllum* in the *gapCp* phylogeny (Fig. 4, 0.99 PP, 63% BS). Variant S falls in with diploid *P. sodiroi* in the *gapCp* tree. Again, indel characters in *pgiC* help further resolve the relationships here: A deletion in *pgiC* intron 15 not found in any other species is shared by variant S and *P. sodiroi*, supporting the hypothesis that *P. sodiroi* is a progenitor and diploid *P. paramicola* is not involved in the origin of the páramo tetraploid (Fig. 4). Diploid *P. orbiculatum* accessions share a deletion which is not present in *P. sodiroi* or the páramo tetraploid. This is consistent with earlier work concluding that diploid *P. orbiculatum* is not a likely progenitor of the páramo tetraploid cytotype (Lyons, McHenry, and Barrington, 2017). The chloroplast phylogeny suggests *P. polyphyllum* as the maternal progenitor, but the node support for this relationship is low (0.64 PP). Building on earlier work (Barrington, 1990; Lyons, McHenry, and Barrington, 2017), we have identified the missing progenitor "X" as *P. polyphyllum* and the missing progenitor "Y" as *P. sodiroi*, and now are able to denote the páramo tetraploid genome as PPSS (Fig. 2; Fig. 5).

The páramo tetraploid is exindusiate and occurs at the highest elevations in the Central American highlands, undocumented below 3400 m (Barrington, 2011). Its genetic and morphological affinities to the páramo clade corroborate the hypothesis that it is of exclusively Andean heritage (Barrington, 1990). McHenry and Barrington (2014) placed this species in the páramo clade along with diploids *P. polyphyllum* and *P. sodiroi*. *Polystichum sodiroi* was initially suggested as a putative progenitor for the páramo tetraploid in Barrington (1990). Here, two closely related but genetically distinct Andean páramo diploids emerge as progenitors for the páramo tetraploid.

It should be noted that significant taxonomic issues surround the species we here call the páramo tetraploid. The name applied to most small exindusiate *Polystichum* species from the páramos in Latin America, *P. orbiculatum*, has been applied to the páramo tetraploid in the Talamanca, including in recent treatments (Barrington, 1990, 2011; Little and Barrington, 2003; Driscoll and Barrington, 2007; McHenry and Barrington, 2014). Especially earlier, the name *P. polyphyllum* was applied to these same plants. Neither of these names is appropriate for the widespread páramo tetraploid, because they both have strictly 2-pinnate fronds with acute pinnae rather than fronds with some pinnatisect segments and attenuate pinnae. The first name (*P. orbiculatum*) relates to a diploid species common in the central Andean páramos; this species is not involved in the origin of the páramo tetraploid (Lyons, McHenry, and Barrington, 2017). The latter name (*P. polyphyllum*) is now restricted to one of the diploid progenitors of the páramo tetraploid, which is most common in the northern Andean páramos. These names, and an array of other combinations considered, appear to be inappropriate for the páramo tetraploid for a variety of reasons, and thus a new name for the *Polystichum* páramo tetraploid is under consideration.

Patterns in polyploid speciation.—The Costa Rican Cordillera Talamanca is host to a unique assemblage of *Polystichum* species, combining the floristic influences of the Mayan and Andean diversity hotspots. In this distinctive biogeographic context, we have demonstrated polyploid speciation resulting in new lineages derived from the Andean and Mayan clades. The polyploid species incorporate the legacy of the two regions into their genomes, forming new lineages with novel genotypes. A picture of a reticulate species complex is emerging from the constantly evolving data sets we are building for these entities; it seems that the implicated diploids are contributing repeatedly to nascent polyploid lineages.

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APPENDIX: Sources of material for DNA extraction. Specimens at VT unless otherwise noted. Sequence within accession: ***Taxon***: *Voucher specimen*; GenBank accession (*rbcL*, *rps4*, *rps4-trnS*, *trnLF*, *gapCp*, *pgiC*).

Polystichum alfaroi (Christ) Barrington: *S. A. Jorgensen 901*; MW000297, MW000289, MW000265, MW000256, MW000307, MW000273. ***Polystichum concinnum*** Lellinger ex Barrington: *D. S. Barrington 2292*; MW000298, MW000290, MW000266, MW000257, MW000323, MW000276. ***Polystichum dubium*** (Karst.) Diels: *M. Lehnert 1277*; KF020330, KF020450, KF020410, KF020357, MW000324, –. ***Polystichum faucicola*** M.Kessler & Lehnert: *M. Lehnert 1257*; KC819964, KC819994, KC819979, KC819949, MW000325, MW000326, –. ***Polystichum hartwegii*** (Klotzsch) Hieron.: *T. Quedensley 4936*, KF020335, KF020444, KF020400, KF020363, MW000326, –. ***Polystichum lilianiae*** Barrington: *S. A. Jorgensen 903*; MW000299, MW000291, MW000267, MW00025, MW000327, MW000277 MW000275. *S. A. Jorgensen 905*; MW000300, MW000292, MW000268, MW000259, MW000329 MW000328, MW000278. ***Polystichum lonchitis*** (L.) Roth: *J. Schneller s.n.*; –, –, –, –, KX866669, KX786227. *P. Zika 10404*: MW000301, MW000293, MW000269, MW000260, –, –. ***Polystichum muricatum*** (L.) Fée: *D. Little 292*; –, –, –, –, MW000308, –. *D. Little 349*; AF537251, KF020445, KF020404, EF177275, –, –. ***Polystichum paramicola*** Kessler and A.R.Sm.: *J. Mostacero*

1391; MW000303, –, –, MW000264, MW000312, MW000288. ***Polystichum* páramo tetraploid**: *D. S. Barrington 2143*; KC878853, KF020456, KF020419, KC878863, MW000322, MW000284 MW000286. *D. S. Barrington 2145*; MW000304, MW000294, MW000270, MW000261, MW000309 MW000310, MW000283 MW000285. ***Polystichum platyphyllum*** (Willd.) C.Presl: *M. A. McHenry* 10-55; KC878854, KC890815, KF020426, KC878864, MW000313, KY610523. ***Polystichum polyphyllum*** (C.Presl) C.Presl: *M. A. McHenry 10-51*; KF020342, KF020455, KF020418, KF020374, MW000314, KY610522. ***Polystichum sodiroi*** Christ: *M. A. Sundue 1060*: KF020346, KF020435, KF020378, KF020469, MW000315, MW000287. ***Polystichum speciosissimum*** (Kunze) R.M.Tryon & A.F.Tryon: *D. S. Barrington 2150*; –, –, –, –, MW000316, –. *D. Little 297*; AF537255, KF020443, KF020397, EF177317–, –. ***Polystichum talamancanum*** Barrington: *J. Kluge 1435*; MW000305, MW000295, MW000271, MW000262, MW000321 MW000320, MW000279 MW000281. *J. Kluge 1445*; MW000306, MW000296, MW000272, MW000263, MW000318 MW000317, MW000280 MW000282. ***Polystichum turrialbae*** Christ: *D. Little 295*; AF537259, KC890818, KF020403, KC878865, MW000319, MW000274. ***Polystichum wolfii*** *Hieron.*: *D. Little 300*; MW000302, –, –, –, –, –. *M. A. McHenry 09-03*; –, –, –, –, MW000311, –.

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Botrychium furculatum (Ophioglossaceae), a New Moonwort Species from the Rocky Mountains of North America

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Abstract.—***Botrychium furculatum*** S. J. Popovich & Farrar is a new species widespread in the central and southern Rocky Mountains from Alberta and Montana south to New Mexico, with additional populations in the Cypress Hills of Saskatchewan and the Black Hills of Wyoming and South Dakota. Based on allozyme banding patterns, it is inferred to be an allotetraploid with *B. pallidum* W. H. Wagner as one parent and another, as yet undescribed, diploid species (*B.* "farrarii") as the other parent. Genetically, *B. furculatum* is distinguished from *B. pallidum* by exhibiting fixed heterozygous loci in which expressed alleles of one of the genomic contributions matches those of *B. pallidum*, whereas many alleles of the other genomic contribution have not been detected in *B. pallidum*, but are displayed by *B.* "farrarii." Morphologically, a suite of leaf characters differentiates *B. furculatum* from *B. pallidum*, particularly a more pronounced bowed or wishbone-like junction of sporophore and trophophore. Gradations in morphology and color between the two species have led to erroneous reports of *B. pallidum* in the Rocky Mountains. A key to differentiate *B. furculatum* from similar species is presented.

Key Words.—*Botrychium*, allozymes, Rocky Mountain flora, allotetraploid speciation

W. H. "Herb" Wagner was exceptional in his ability to recognize diagnostic morphological characters differentiating cryptic species within *Botrychium* s.s. As a result, he and Florence Wagner from 1981 through 2002 described 16 of the 27 taxa known in North America at that time. Herb and Florence also made initial studies on several more putative taxa, often assigning them informal names. They applied the provisional specific epithet "adnatum" to a group of plants collected by Peter Lesica in 1996 in two prairies of western Glacier National Park, Montana, the name reflecting the broad, sometimes decurrent, attachment of pinnae to the rachis in those populations. In an unpublished 1998 field study of these sites, the Wagners (accompanied by

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Gilman) examined *B.* "adnatum" and also what they believed to be the diploid species *B. pallidum* and *B. simplex* in the same area. They considered a possible allopolyploid origin of "adnatum" through hybridization between those two species and sent specimens of each taxon to Farrar for genetic analysis of this possibility via enzyme electrophoresis.

Based on the allelic composition of "adnatum" at 20 gene loci and the presence of fixed heterozygosity at most loci, Farrar (unreported prior to this paper) concluded that "adnatum" was probably an allotetraploid and that *B. pallidum* was likely one of its parents. However, the non-*pallidum* complement of alleles detected in "adnatum" did not match those of *B. simplex*. Farrar also concluded from allozyme analysis that the plants the Wagners had identified as *B. pallidum* were not that species. Rather, a preponderance of fixed heterozygosity in their allelic constitution also indicated allopolyploidy and matched that of *B. gallicomontanum*, an allopolyploid with *B. pallidum* as one of its parents (Farrar and Johnson-Groh, 1991). Because of these genetic results, and because of a perceived failure to find additional populations, formal publication of "adnatum" was withheld.

In 2005, in cooperation with Glacier National Park botanists, we initiated further study of "adnatum" at the original localities, and a broader search for this taxon in the Rocky Mountains of southern Canada, Colorado, and the Black Hills of South Dakota. Field visits and genetic analysis of the Glacier National Park plants confirmed Farrar's initial conclusions, and plants of similar morphology and yielding identical genetic profiles were also detected in southern Alberta, central Colorado, and in the Black Hills. Surprisingly, we found that many of the Colorado plants with the allelic composition of "adnatum" correlated in their morphology, pallid appearance, and location to plants reported by Wagner and Wagner (1990) as Colorado paratypes of *B. pallidum*. Less pallid plants in Colorado of the same morphology had been given the provisional name "Colorado" by Peter Root (affiliated with the Denver Botanic Gardens) and were thought to be possibly a form of *B. minganense*. Also in 2005, plants resembling *B. minganense* but with the genotype of "adnatum" were detected at Redbank Spring in the Black Hills of South Dakota and informally named "redbank." Subsequently, Popovich continued survey efforts in Colorado and other western states through the following decade while a Botanist for the US Forest Service. Other Forest Service Botanists and moonwort enthusiasts augmented those surveys and collections.

With increasing familiarity regarding the range of morphologies associated with this genotype, subsequent collections revealed morphologies additional to "adnatum", "Colorado" and "redbank," including plants much larger and plants lacking conspicuously adnate pinnae. In retrospect, it is clear that Wagner and others did see populations of "adnatum" outside of its original site in Glacier National Park but did not recognize them as the same taxon. Because of the widely varying and not universally adnate morphology of this taxon,

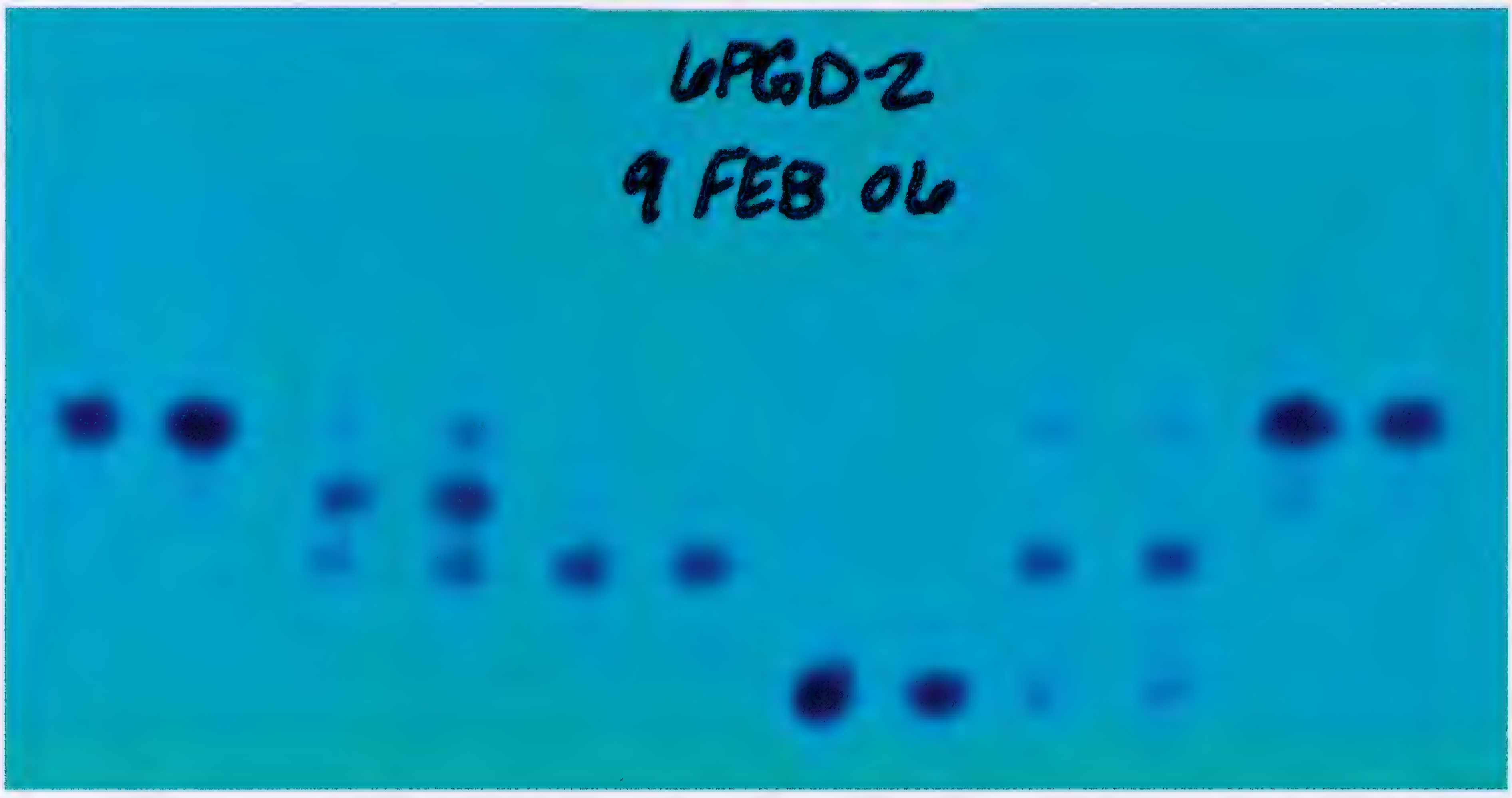

FIG. 1. Typical electrophoresis banding patterns of the enzyme 6-pgd displaying 3 banded allelic patterns in allopolyploid *Botrychium* taxa combining single allele patterns of their diploid parents. From left to right, *B. campestre* (diploid), *B. campestre* × *B. tunux* (probable allotetraploid, undescribed), *B. tunux* (diploid), *B. crenulatum* (diploid), *B. crenulatum* × *B. campestre* (allopolyploid *B. ascendens*), *B. campestre* (diploid).

Farrar and Popovich (2012) applied a different informal name, *B.* "furculatum," which we formalize below as *B. furculatum* S. J. Popovich & Farrar.

Important range extensions for this species were added in southern Saskatchewan by the authors and in New Mexico and Wyoming by Ben Legler. In Legler's Wyoming collections sent to Farrar, he included specimens he considered "similar to, but possibly different from *B.* 'furculatum'," provisionally naming these other plants *B.* "farrarii." Farrar's genetic analyses of those plants indicated not only were they a new diploid taxon, but one possessing the alleles of *B. furculatum* not contributed by *B. pallidum.* A formal description of *B.* "farrarii" is in progress.

Recognition of allopolyploidy as a significant element in understanding *Botrychium* evolution began with cytological determination by W. H. Wagner (1955) of "doubled" chromosome numbers in *B. matricariifolium* and what he later concluded (Wagner and Lord, 1956) was *B. minganense.* As subsequent publications by Wagner and co-workers added taxa to the list of *Botrychium* allopolyploids, detections of their probable diploid parents were greatly aided by genetic evidence derived through enzyme electrophoresis (*e.g.,* Hauk and Haufler, 1999; Farrar and Johnson-Groh, 1991; Stensvold, Johnson-Groh, and Farrar, 2002; Stensvold and Farrar, 2016), a technique revealing sets of alleles distinctive to each diploid and with both parental contributions being visualized in derived polyploids (see Materials and Methods, and Fig. 1). Inclusion of allozyme-confirmed taxa in chloroplast DNA analyses of all known species (Dauphin *et al.*, 2017) allowed detection of the maternal parent of each polyploid taxon through alignment with that diploid. Lastly, development of procedures allowing visualization of codominant nuclear markers (Dauphin *et al.*, 2018) produced results congruent with morphology, cytology, allozymes, and chloroplast DNA in depicting an array of 15 allotetraploid and one allohexaploid derived from 10 diploid taxa from the three principal clades of *Botrychium* (Dauphin *et al.*, 2018) with only the diploids *B. tunux* and five species of the *B. simplex* subclade excluded from participation in allopolyploid parentage.

MATERIALS AND METHODS

Samples of putative *B. furculatum* were collected by the authors and other field botanists from sites in Alberta, Saskatchewan, Montana, Wyoming, South Dakota, Colorado, and New Mexico and sent to the Farrar laboratory at Iowa State University for genetic analysis and herbarium curation. For use in genetic analyses, each specimen and each collection were assigned a unique tracking code. These codes, appearing in our figures as numbers attached to individual plants, indicate genetic verification of the identification.

Morphological measurements were taken from live plants, from dried, pressed specimens, and from high-resolution color scans of pressed specimens enlarged to optimize measurement accuracy. Depending upon attribute examined, between 82 and 256 pressed individuals from across the range were measured. Qualitative characters that are not well-captured in pressed specimens, *e.g.*, longitudinal folding of the trophophore, fresh color and glaucousness, and the orientation ("bowing out") of the trophophore stalk at its junction with the sporophore stalk, were scored on living or freshly collected specimens. Spore sizes were measured in the longest diameter on spores mounted in Hoyer's chlorohydrate clearing solution. A minimum of 20 spores was measured for each sample plant (n=40 plants for *B. furculatum*, 15 for *B. pallidum*, and 14 for *B.* "farrarii") selected from populations representing the range of *B. furculatum*. Spores were extracted from multiple sporangia for each plant and measured under a compound microscope.

Enzyme electrophoresis followed procedures described in Stensvold and Farrar (2016). Patterns of allele distributions were analyzed for 269 plants of *B. furculatum*, 175 plants of *B. pallidum* and 18 plants of the rare diploid, *B.* "farrarii," each sampled from across its known range. Plants were analyzed for 22 gene loci from 10 enzyme systems using three buffer systems from Soltis *et al.* (1983): buffer system 7 (0.038M LiOH, 0.188M boric acid) for resolving enzyme systems, aspartate aminotransferase (*Aat*) and triose-phosphate isomerase (*Tpi*); buffer system 9 (0.065M L-Histidine, 0.015–0.016M citric acid, anhydrous) for resolving enzyme systems, malate dehydrogenase (*Mdh*), phosphoglucomutase (*Pgm*), 6-phosphogluconate dehydrogenase (*6-Pgd*), and phospho-glucoisomerase (*Pgi*); and buffer system 11 (0.4M citric acid, trisodium salt) for resolving enzyme systems, aconitase (*Acn*), diaphorase (*Dia*), isocitrate dehydrogenase (*Idh*), and shikimate dehydrogenase (*Skdh*). Alleles are expressed as descending numbers relative to distance of migration on the starch gels, with "1" being the most distal from the origin.

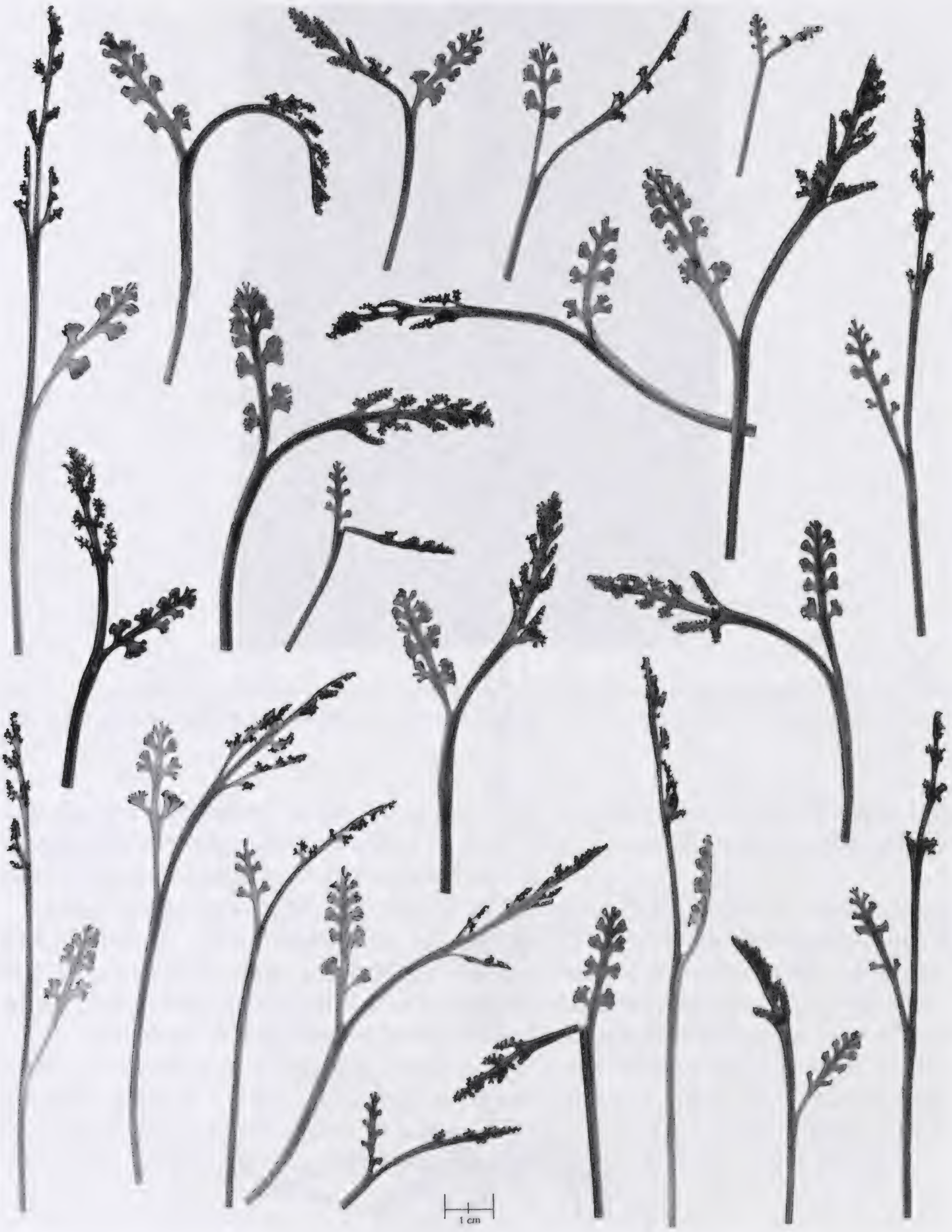

FIG. 2. Specimens of *B. furculatum* from the type locality, Grand County, Colorado.

RESULTS

Morphological measurements and shapes that characterize *B. furculatum* are presented in the species description with diagnostic characters highlighted in italics and shown in Figs. 2–5 and Fig. 7. Relevant comparisons to similar

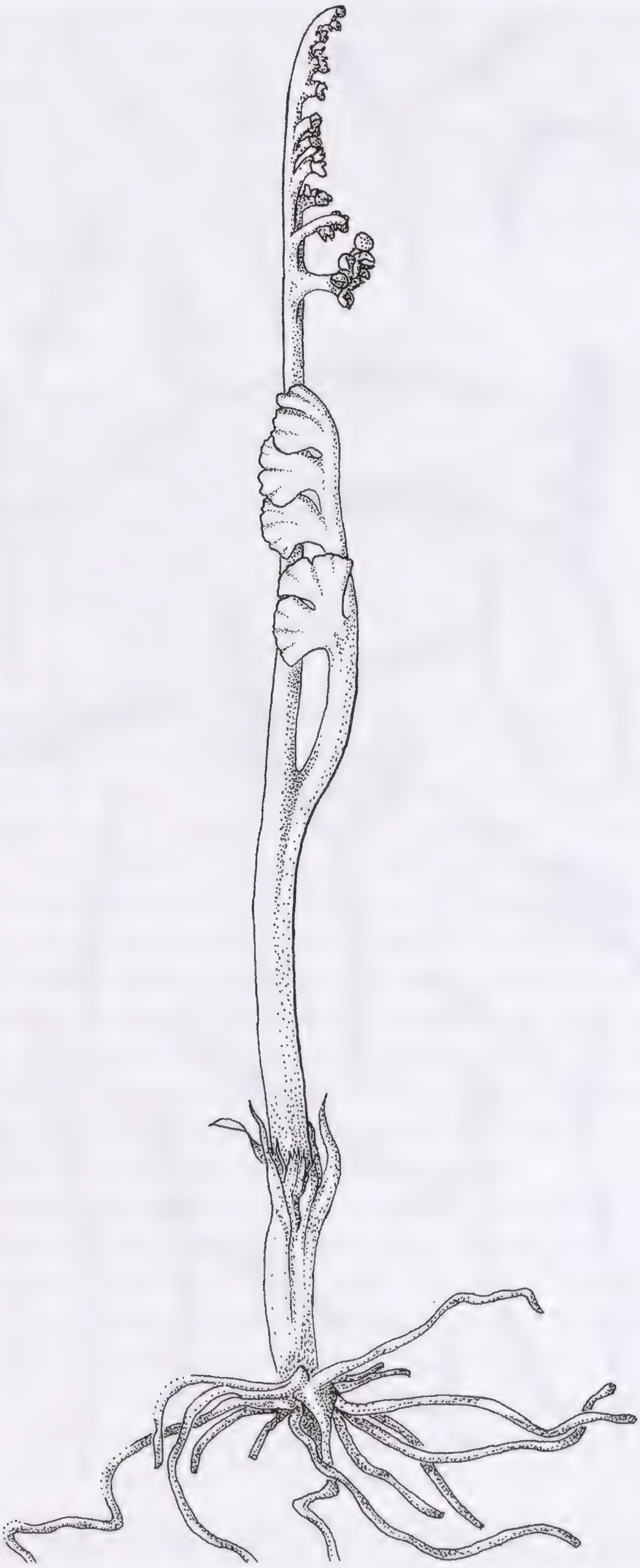

FIG. 3. *Botrychium furculatum*, illustrating in-field stature and morphology. Line drawing by Daryl E. Mergen and modified by the authors. Above-ground leaf from a high-resolution image of a typical plant from near Monarch Pass, Colorado.

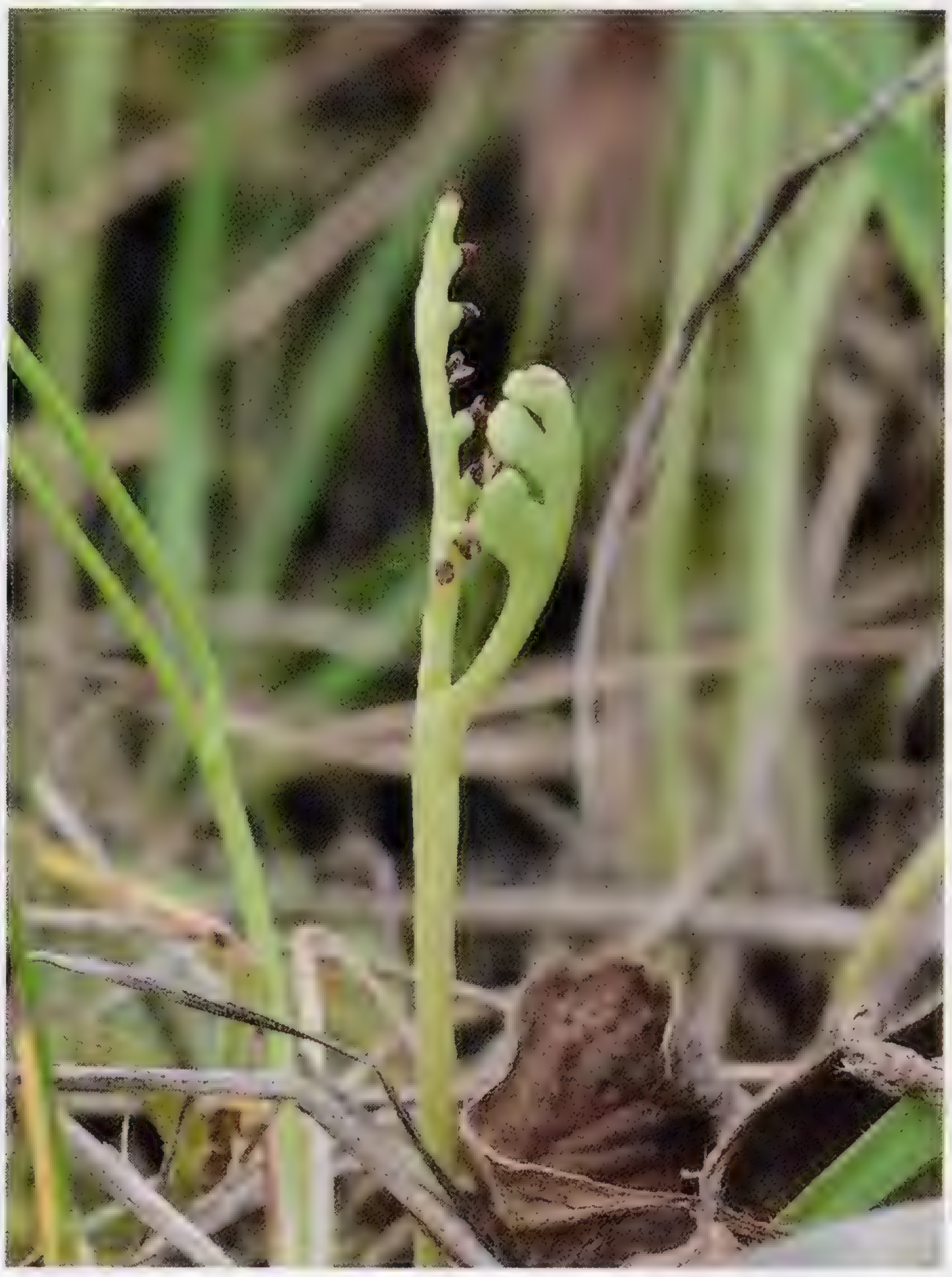

FIG. 4. A small individual of *B. furculatum*, showing trophophore bowed outward at base. This character may be difficult to assess on pressed specimens. Cypress Hills, Saskatchewan. Photo: Glen Lee.

species are presented in the accompanying key and Fig. 6. Additional, less diagnostic, measures not included in the formal description are available from the authors. A range map is provided as Fig. 8.

In electrophoretic analysis of enzyme extracts, both *Botrychium pallidum* and *B.* "farrarii" expressed single alleles at 21 of 22 gene loci and two alleles at one locus each (Table 1). Of 20 loci visualized for *B. furculatum,* only one, *Skdh*, expressed an allele not also present in either *B. pallidum* or *B.* "farrarii." At 11 loci where the expressed allele differed between the diploid taxa, plants of *B. furculatum* expressed heterozygosity combining alleles present in the putative parents. Of the 11 loci not expressing heterozygosity in *B. furculatum*, 9 expressed the single allele that was expressed in both *B. pallidum* and *B.* "farrarii." At two loci no activity was expressed in *B. furculatum* plants.

DISCUSSION

Evidence from morphology and allozymes demonstrates that *B. furculatum* is a new allotetraploid species derived in part from parentage by *B. pallidum*, as first hypothesized by Wagner for plants at Glacier National Park. The pale coloration of the plant in life, particularly as expressed by Colorado populations, and the commonly asymmetrical lobing of the pinnae appear to be directly inherited from that species. This close relationship is also

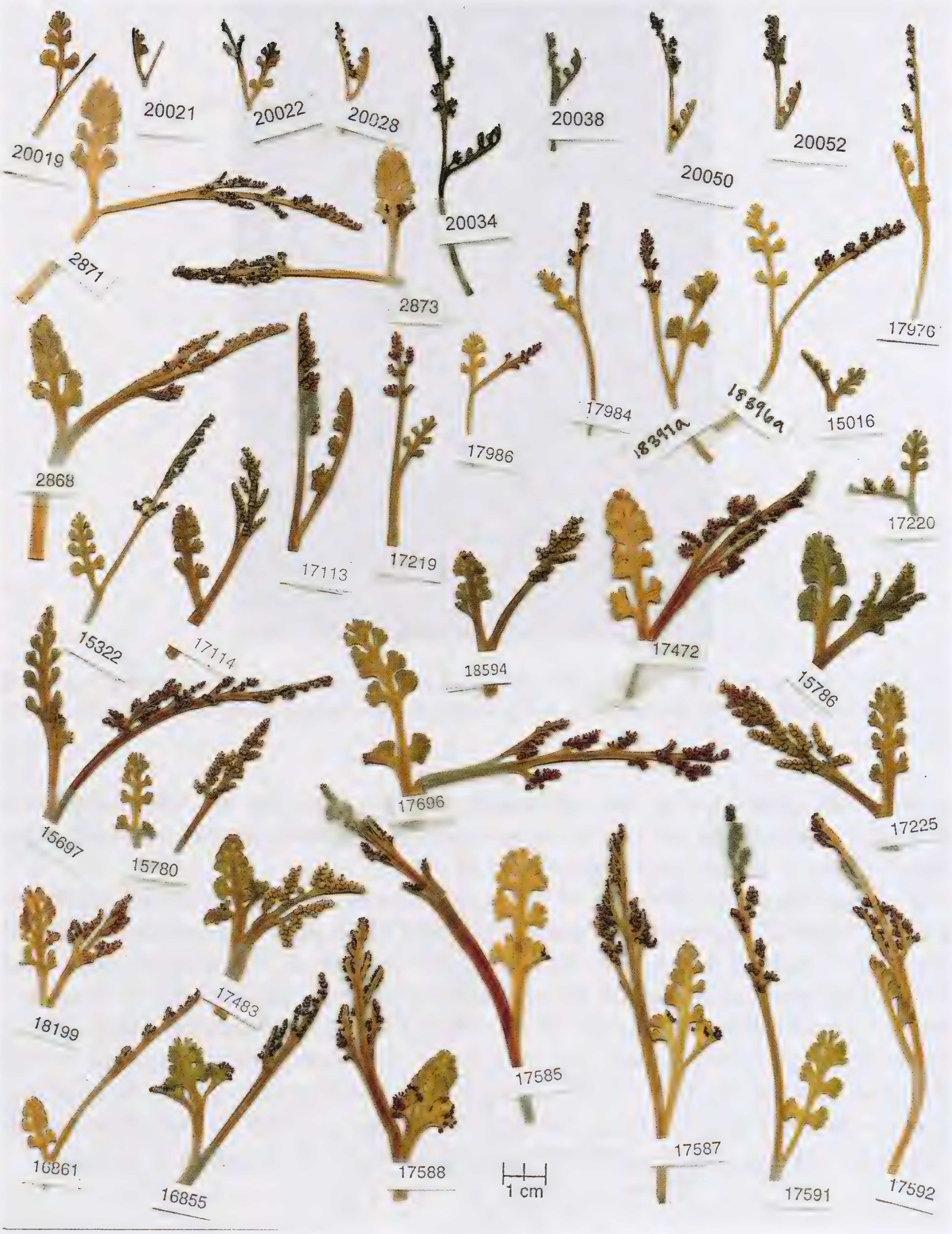

FIG. 5. Range-wide morphological variation in *B. furculatum*. Major sites arranged from north to south across the species range. Numbers attached to specimens were assigned by the Farrar laboratory for tracking individuals during enzyme extraction and analysis; all specimens at ISC. Southwestern Saskatchewan, Cypress Hills Interprovincial Park: 20019, 21, 22, 28, 34, 38, 50, 52 (missing sporophores in 20019 and 21 used for enzyme extraction); Northwestern Montana, Glacier Nat. Park, Big Prairie: 2868, 71, 73; Northern Wyoming, Bighorn Mountains: 17976, 84, 86, 18396a, 97a; Northeastern Wyoming, Black Hills, Warren Peak: 15016, 17220; Western South Dakota, Black Hills: 15322 (Northern Hills), 17219 (Eagle Cliffs), 18594 (Hat Mountain), 17113, 14 (Reynolds Prairie); Central Colorado, Rocky Mountains: 15697 (Boreas Pass), 15780, 86 (Marshall Pass), 17696 (Weston Pass), 17472 (Vail Pass), 17225 (Grizzly Gulch), 18199 (Winter Park), 17483 (Brainard Lake); Northern New Mexico, Vermejo Park Ranch: 16855, 61, 17585, 88, 87, 91, 92.

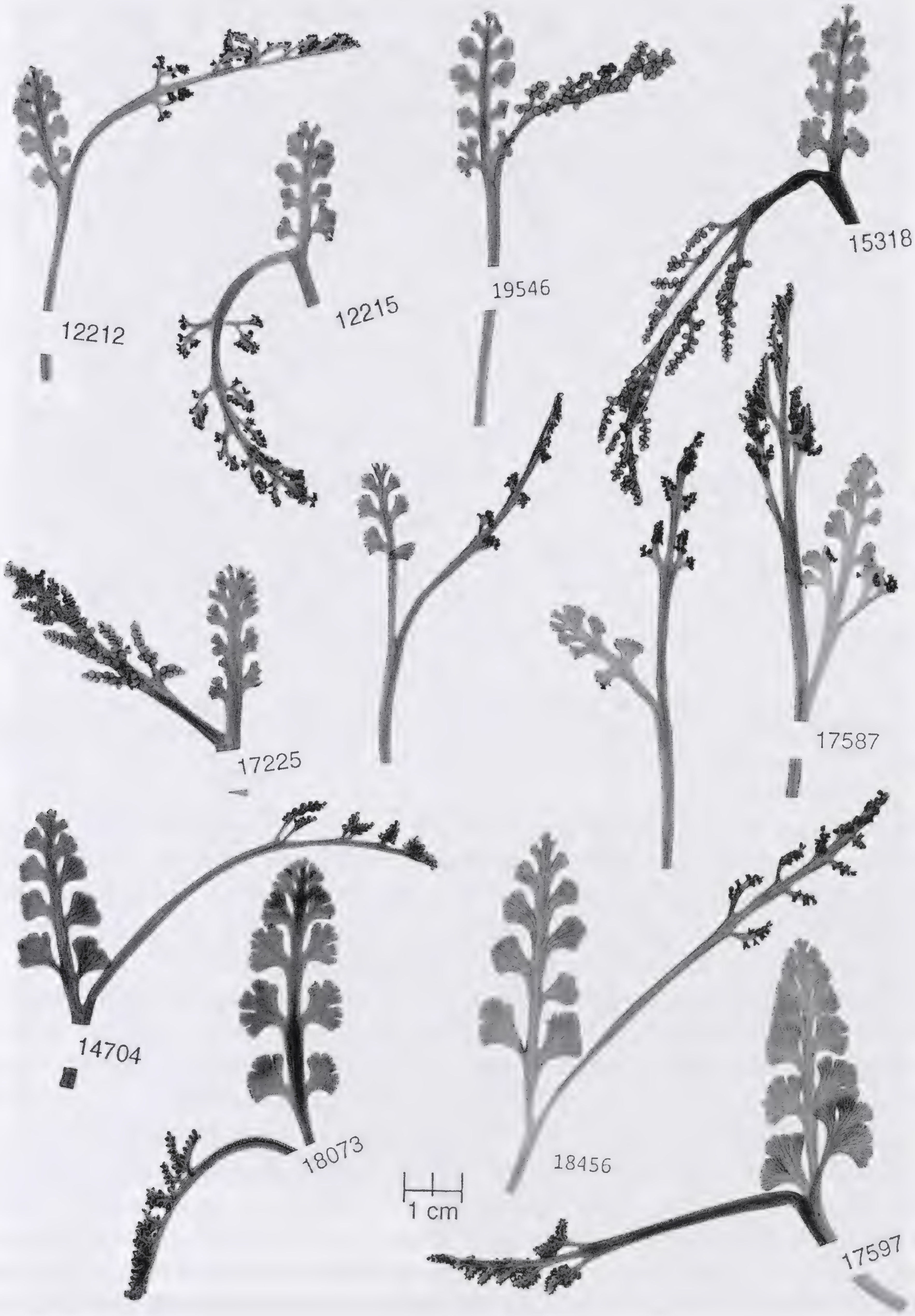

FIG. 6. Comparison of *Botrychium furculatum* to *B. minganense* and *B. pallidum*. Top row: *B. pallidum*: 12212, Elk Island Nat. Park, central Alberta; 12215, Elk Island Nat. Park, central Alberta; 19546, northwestern Minnesota; 15318, Black Hills, South Dakota. Middle row: *B. furculatum*: 17225, Grizzly Gulch, Colorado; (two un-numbered plants) Rocky Mtn. Nat. Park, Colorado (type locality); 17587, Vermejo Park Ranch, northern New Mexico. Bottom row: *B. minganense*: 14704, Rocky Mtn. Nat. Park, Colorado; 18073, Black Hills, South Dakota; 18456, Bighorn Mts., Wyoming; 17597, Vermejo Park Ranch, northern New Mexico.

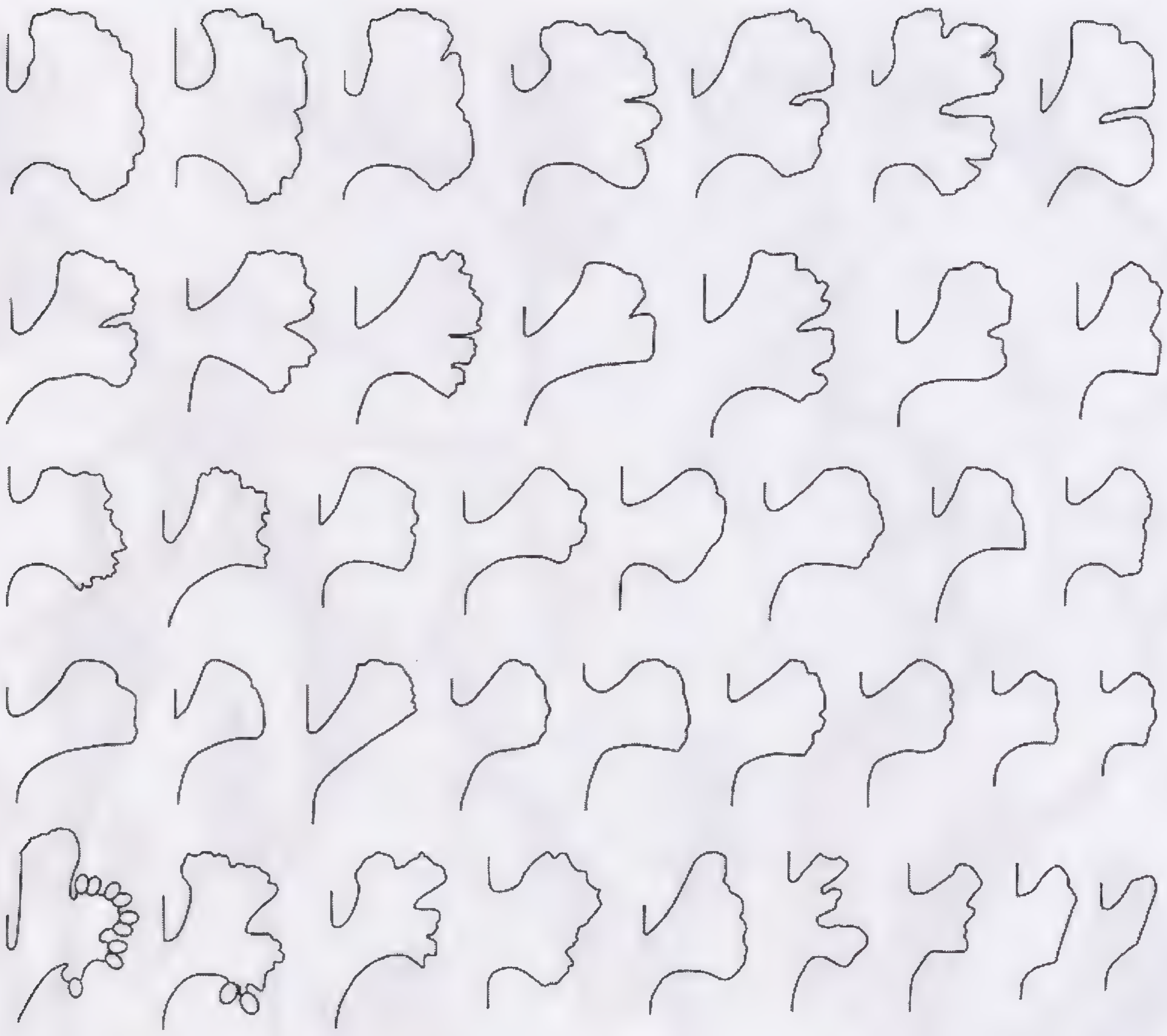

Fig. 7. Outlines of basal pinnae of *B. furculatum* illustrating high variation of lobing, margins, size, and width. Outlines were traced from enlarged, high-resolution images of pressed specimens across the species range exclusive of Canada.

evidenced by the initial identification (Wagner and Wagner 1990) of some Colorado plants as "Rocky Mt. *B. pallidum*." Allozyme evidence also supports parentage by an as yet undescribed diploid, *B.* "farrarii." The adnate pinna attachment of some plants described by Wagner and alluded to in his provisional name "adnatum" may be derived from *B.* "farrarii."

Establishing ploidy level by direct chromosome counts in *Botrychium* is difficult because meiosis, which is the optimum stage of the fern life cycle to observe chromosomes (Manton, 1950), occurs while leaves are emerging from underground and are undetectable or, at best, unidentifiable in the field. Increased spore size and disproportionately high frequencies of heterozygosity provide alternate evidence of allopolyploidy. Although a rigorous comparison of spore size in all *Botrychium* allopolyploids relative to their parent diploids has not been published, a search of published *Botrychium* species descriptions including spore size, supplemented by our own spore measurements, yields an estimate of spore size in 13 allopolyploid species averaging 42.3 μm (39–45), and a 7.1 μm (3.2–11.2) increase in size relative to the average size of their assigned diploid parents (avg. 35.2 μm). The spore size of *B. furculatum,* with an average spore size of 43.0 μm and diploid parent average of 35.2 μm, fits this pattern. Furthermore, in *B. furculatum,* heterozygosity is displayed in most plants at 11 of 22 loci, and no plants were observed without heterozygous loci.

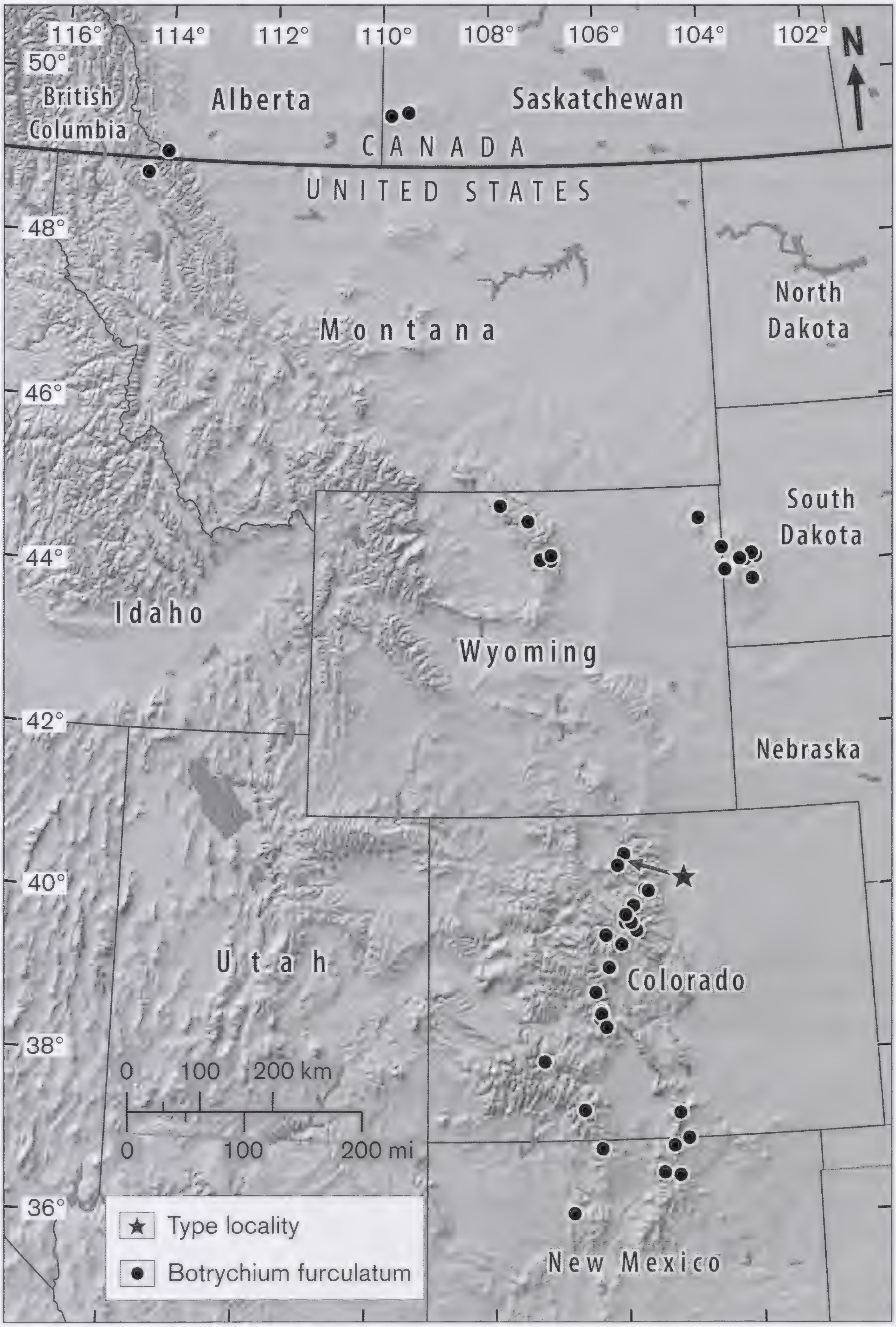

FIG. 8. Range of *Botrychium furculatum*. A dot may represent more than one locality or collection. The star symbol indicates the type locality, offset by arrow.

TABLE 1. Alleles expressed at enzyme-coding loci in *B. furculatum* and its putative ancestral diploid parent species. Allele numbers joined by "+" are alleles contributed by parent taxa expressing different alleles at a given locus. Expression of a single allele in *B. furculatum* (where two are possible) presumably results from failed expression (silencing) of one of its two inherited loci. Allele numbers reflect relative migrating positions within genus *Botrychium*. A "null" listing reflects characteristic non-expression of enzyme activity at a locus for a given taxon.

Locus	***B. pallidum***	***B. furculatum***	***B.* "farrarii"**
	n = 175	n = 269	n = 18
Aat-1	1	1+3 or 3	3
Aat-2	3	3	3
Aat-3	2	1.5+2 or 2	1.5
Aat-4	3	1.5+3 or 3	1.5
Acn-1	1	–	2
Acn-2	2 or 3	3	3
Dia-1	2	2+4 or 4	4
Dia-2	1	1	1
Dia-3	null	3	3
Dia-4	8	7.5+8 or 7.5 or 8	7.5
Idh-1	1	1+4 or 4	4
Mdh-1	1	1	1
Mdh-2	3	3+4.5 or 3	4.5
Mdh-3	2	2	2
Mdh-4	2	–	null
6Pgd	1	1	1
Pgi-2	2	1.6+2 or 2	1.6
Pgm-1	1	1+3 or 3	3
Pgm-2	2	2+4 or 2	2 or 4
Skdh	1	1+3 or 1 or 3	1
Tpi-1	3	3	3
Tpi-2	3	3	3

Co-dominant allele expression at heterozygous loci in allotetraploid species provides evidence of their probable diploid parentage. If at least one allele at each locus is contributed by each parent, a majority of alleles present in the allopolyploid should be present as well in one or the other of the proposed diploid parents. In *B. furculatum*, alleles at 19 of 20 loci expressing activity are present in either *B. pallidum* or *B.* "farrarii" (Table 1). One allele, *Skdh*-3, was not detected in either putative parent although it is present in several diploid *Botrychium* species at a low frequency and may yet be detected in further sampling of *B. pallidum* or *B.* "farrarii." Many of the alleles expressed in *B. pallidum* are common among diploid *Botrychium* and individually do not exclude other species from consideration, but the consistent expression of *B. pallidum* alleles in the 20 expressed loci of *B. furculatum* constitutes strong support for its parentage of *B. furculatum*. Alleles of *B.* "farrarii" are also expressed in each of the 20 visualized loci of *B. furculatum*, and its lack of expression at *Mdh*-4 is also shared with *B. furculatum*. In our analyses of North American *Botrychium*, the alleles *Dia*-4 = 7.5, *Mdh*-2 = 4.5, and *Pgi*-2 = 1.6 have been detected only in *B.* "farrarii" and *B. furculatum*. The presence of these private alleles in *B. furculatum* strongly supports the hypothesis of its parentage by *B.* "farrarii."

To determine the female (chloroplast donor) parent of *B. furculatum* we included allozyme-determined plants of all *Botrychium* species in a worldwide phylogenetic study (Dauphin *et al.*, 2017) using chloroplast DNA sequencing. Results clustered *B. furcul*atum with *B.* "farrarii" indicating that species to be the female parent of *B. furculatum.*

Current knowledge, including information on morphology, allozymes, nuclear and chloroplast DNA, ploidy, parentage, range, and habitats provides convincing evidence that *B. furculatum* warrants recognition as a new allotetraploid species.

TAXONOMIC TREATMENT

Botrychium furculatum S. J. Popovich & Farrar, sp. nov.

TYPE: USA. Colorado: Grand County, Rocky Mountain National Park, Kawuneeche Valley, Lulu City townsite, 40°26′48.47″N, -105°50′51.40″W, 2857m, common in herb-graminoid forest opening along Colorado River trail, with congeners *B. echo, B. hesperium, B. lanceolatum,* and *B. minganense*, 17 Aug 2011, *Popovich 8580 & Connor* (holotype: COLO!; isotypes: ALTA!, BRIT!, CAN!, CS!, ID!, ISC!, KHD!, LM!, MICH!, MO!, MONT!, NY!, Rocky Mtn. Nat. Park working herbarium!, RM!, US!, VT!, WTU!). FIGS. 2–4.

Etymology.—Latin *furcula*, little fork, the zoological term for the avian wishbone, referring to the bowed junction of the sporophore and trophophore. A suitable common name is wishbone moonwort.

Diagnosis.—Differs from other members of the *Botrychium* "Campestre clade" in its combination of pallid color, irregular pinna outline, and trophophore bowed out at base.

Description.—Perennial herb, producing a single aboveground leaf annually, this divided dichotomously into a sterile, laminate *trophophore* and a fertile, spore-bearing *sporophore*, dying back following spore release; plant endomycorrhizal.

Underground stem erect, unbranched, bearing 5–18+ fleshy roots up to 1.2 mm wide; spherical gemmae sparingly present or absent, 0.5–1.0 mm in diameter, attached directly to the stem. Depth from soil surface to belowground apical bud 2.3–7.4 cm.

Aboveground leaf erect, fleshy, glabrous to glaucous; color variable, pale green, whitish-green, or yellow-green to extremely pale yellow-white, rarely glaucous blue-green; total leaf height from ground to top of sporophore (1.7) 4–10 (16.4) cm; common stalk (petiole) (0.7) 1.5–6.5 (8.8) cm long, (0.3) 0.5–1.5 (5.0) times the length of the mature sporophore; *trophophore stalk often outwardly bowed, joining sporophore stalk in a spreading to narrow wishbone-like* "junction".

Trophophore (0.6) 1.2–3.6 (5.3) cm long; *stalk (0.1) 0.3–1.4 (2.9) cm long, (0.3) 0.7–2.1 (3.8) times the entire length of the rachis;* blade erect, (0.4) 0.7–2.3

(4.4) cm long, (0.2) 0.5–1.0 (1.7) cm wide at basal pinnae, narrowly ovate-oblong to ovate or linear-oblong in outline, once pinnate, *more or less folded longitudinally and trough-like in life* with apex prow-like and inflexed to 90° toward sporophore.

Pinna pairs (2) 4–5 (7), *proximal 1–2 pairs usually clasping the sporophore stalk at maturity.* Pinnae ascending to spreading, remote to approximate. Lower pinnae usually well-spaced with upper pinnae approximate to overlapping, distance between basal and 2nd pairs (0.6) 1.8–6.5 (11.5) mm, distance between 2nd and 3rd pairs (0.3) 0.5–3.0 (5.8) mm, the former distance (1.0) 1.2–3.0 (5.0) times the latter; basal pinnae usually largest (0.6) 1.8–5.0 (9.0) mm long, (0.5) 1.6–4.8 (7.5) mm wide, subsequent pairs gradually reduced in size and lobing, short-stalked to adnate, sometimes decurrent; pinna margins not overlapping the rachis.

Pinna outline fan-shaped to spatulate, sometimes ± rhombic, often asymmetric, widest at outer margin, *span (24) 70–120° (148)*, 50–70° in small plants; pinna palmately lobed to entire, *lobes often asymmetric and shallow, sometimes cleft ± halfway to pinna base; principal lobes usually two, spreading, with upper lobe usually larger (or longer) and more developed*; sinuses usually rounded, sometimes angular in deeper lobing; *pinna and lobe outer margins irregularly crenate to erose or lacerate,* tending toward ± entire in small plants; pinna side margins slightly to moderately concave, sometimes strongly concave-recurved or more or less straight; junction of outer and side margins usually rounded, occasionally sharp-cornered; midrib lacking, veins dichotomous, 2 or 4 (6) major veins entering the pinna base, (4) 11–31 (45) ending near the margins. *Often some pinnae appearing malformed, unevenly truncate, or terminating abruptly as if cut, or stubby. Stalks of lower pinnae (1/6) 1/3–2/3 (9/10) as wide as the pinnae.* Basal pinnae occasionally bearing sporangia on their margins.

Sporophore erect, sometimes arching away from the trophophore, (1.0) 1.9–7.6 (10.3) cm long, *(1.0) 1.4–2.6 (3.4) times the length of the trophophore*; stalk (0.2) 0.8–3.9 (6.1) cm long, *(0.2) 0.5–1.3 (1.9) times the entire length of the trophophore*; sporangia-bearing portion branched, narrowly ovate to linear in outline, 1–2-pinnate (2-pinnate usually restricted to lowermost branches); branches in 4–9 (11) pairs (rarely absent in small plants with sessile sporangia), usually short-stalked to sessile, 0.2–11.0 mm long, strongly ascending, *twisted inward in life and appressed to the rachis such that sporangia often partially obscure the rachis*; lowermost branches the longest, *often conspicuously so in large plants,* to 4.6 cm long and 0.8 times the rachis length; proximal branchlets of basal branches often descending; *sporophore often terminating in an unbranched rachis bearing 2–7 (10) sessile sporangial clusters*; sporangia (8) 40–150 (200+), 10–70 in small plants, partially embedded, *not crowded*, dull yellow at spore release, opening in June at low elevations through early August at high elevations; *spores (39) 41–45 (49) avg. 43.02 μm in longest diameter.* Apparently tetraploid.

Habitat.—Open montane to subalpine meadows, forest openings, and high-latitude prairies, especially where exhibiting historic disturbance by natural or anthropogenic processes, *e.g.* fire scars, snow slides, retired mining districts, roadsides and ski runs; often in older (>20 years) disturbed areas where vegetational succession has proceeded to dominance by perennial herbs and grasses but not to canopy closure by woody plants. Elevation ranges from 1235m in the Cypress Hills of Saskatchewan (*Popovich & Farrar FL160716*, ISC!) to 3609m in New Mexico (*Legler 11522*, RM!).

Distribution.—Populations of *B. furculatum* have been morphologically and genetically confirmed in the central and southern Rocky Mountain region of western North America from Alberta, Canada, south to Montana, Wyoming, Colorado, and New Mexico, USA, with easterly outlying populations in the Cypress Hills of Saskatchewan, Canada, and the Black Hills of Wyoming and South Dakota, USA. FIG. 8.

Wagner and Wagner (1990) listed paratype specimens of *B. pallidum* from Colorado. We examined images of cited collections from Boulder County (*W. H. Wagner 84204, 84205*, MICH-images!) and found them to exhibit the morphologies of *B. furculatum*. We did not locate *Root-89-11* from El Paso County. Our collections from Wagner's paratype areas also display the morphologies as well as genotype patterns of *B. furculatum*. *Botrychium pallidum* reported from Colorado by Heil *et al.* (2013) is *B. campestre* (*Stewart s.n.*, COLO!). Barring further evidence, we suggest that *B. pallidum* be excluded from the Colorado flora.

Conservation.—With more than 65 known localities (45 in Colorado alone), many of them with populations numbering 100's of individuals, we believe that *Botrychium furculatum*, although perhaps locally rare in portions of its range, is secure in viability overall. Fortunately, many populations occur on managed or protected public lands, including Waterton-Glacier International Peace Park, Cypress Hills Interprovincial Park, Glacier National Park, Rocky Mountain National Park, and the following National Forests: Arapaho-Roosevelt, Bighorn, Black Hills, Carson, Gunnison, Pike-San Isabel, Rio Grande, Routt, Santa Fe, and White River.

Additional Specimens Examined.—CANADA. Alberta: Waterton-Glacier International Peace Park, Red Rock Canyon, 23 Jun 2005, *Farrar FL050623* (ISC). Saskatchewan: Cypress Hills Interprovincial Park ("CHIP"), Centre Block, Ben Vannock Dr SW of Visitor Centre, 15 Jul 2016, *Popovich & Farrar FL160715* (ISC); CHIP, West Block, Battle Creek Ranger Station Rd, 16 Jul 2016, *Popovich & Farrar FL160716* (ISC).

UNITED STATES. Colorado: Boulder Co., Roosevelt National Forest ("RNF"), Brainard Lake, 15 Jun 2006, *Popovich 8352–8355* (ISC); 29 Jul 2009, *Popovich & Farrar et al. s.n.* (COLO); RNF, Coney Flats, 11 Aug 1999, *Steinmann s.n.* (KHD); Cty, Grassy Top S of Ward, 15 Jun 2009, *Steinmann 2009-2* (ISC). Chaffee Co., San Isabel National Forest ("SINF"), Boss Lake, 10 Jul 2007, *Popovich 8494c* (ISC); SINF, Monarch Ski Area, talus slope, 27 Jul 2009, *Popovich & Farrar FL090727* (ISC); SINF, North Fork Reservoir, natural bank, 20 Jul 2006, *Popovich & Farrar FL060720* (ISC); SINF, Old Monarch Pass, summer 2007, *Kirkpatrick s.n.-4* (ISC). Clear Creek Co., Arapaho National Forest ("ANF"), Eisenhower Tunnel, 22 Jul 2010, *Popovich s.n.* (ISC); ANF, Grizzly Gulch, 10 Jul 2009, *Popovich 8551, 8552* (ISC); ANF, Jones Pass, 28 Aug 2008, *Popovich 8527* (ISC). Conejos Co., Rio Grande National Forest, 2 mi W of Stunner Campground, 13 Jul 1991, *Root 91-28* (KHD). Costilla Co., Vermejo Park Ranch ("VPR"), Culebra Range, NE side State Line Peak, 20 Jul 2009, *Legler 11522* (RM); VPR, Culebra Range, head of W Frk Costilla Crk, 24 July 2009, *Popovich & Farrar FL090724* (ISC); VPR, 24 Jul 2009, *Popovich & Farrar FL090724* (ISC). Grand Co., Rocky Mountain National Park, Kawuneeche Valley, Lulu City townsite, meadow in forest opening, type locality, 14 Jul 2012, *Popovich 8587* (COLO, ISC; genetically confirmed topotype); ANF, St. Louis Creek Rd, 5 Aug 2009, *Smith s.n.* (ICS); ANF, Winter Park Resort, Jack Kendrick ski run, 20 Aug 2008, *Popovich 8535, 8536* (ISC). Gunnison Co., Gunnison National Forest ("GNF"), W side Cottonwood Pass, 15 Jul 2008, *Popovich 8525* (ISC). Hinsdale Co., GNF, W of Slumgullion [Pass] Campground, 19 Aug 1999, *Root 1253* (KHD). Huerfano Co., SINF, Culebra Range, Blue Lakes, 28 Jul 2019, *Popovich & Olson 04* (COLO). Jackson Co., Routt National Forest ("RNF"), Cameron Pass, rest area, 8 Aug 2006, *Popovich 8377–8389* (ISC); RNF, Hi Ho Mine, 15 Sep 2008, *Proctor 80915-1* (ISC); RNF, Medicine Bow, 16 Sep 2008, *Proctor s.n.* (ISC); RNF, switchback on Forest Service Rd 758, 31 Jul 2009, *Popovich & Farrar FL090731* (ISC). Park Co., Pike National Forest ("PNF"), E side Weston Pass, 28 Jul 2009, *Popovich & Farrar FL090728* (ISC); PNF, Guanella Pass, Duck Creek, 5 Aug 2009, *Popovich 8364–8366* (ISC); PNF, above Leavick townsite, 10 Aug 1990, *Root 90-88* (COLO). Pitkin Co., White River National Forest ("WRNF"), Snowmass Ski Area, 29 Aug 2010, *Elliott 1612a* (RM). Saguache Co., GNF, Marshall Pass, 13 Jul 2007, *Popovich 8505a-g* (ISC). Summit Co., PNF, Boreas Pass, Windy Point, 18 Jul 2007, *Popovich & Farrar FL070718* (ISC); WRNF, Vail Pass, old burn scar, 18 Jul 2009, *Popovich & Farrar FL090718* (ISC). Montana: Flathead Co., Glacier National Park ("GNP"), Big Prairie, burned 1988, 26 Jun 1996, *Lesica 7251* (MICH-image!); 19 Jun 1998, *W. H. Wagner s.n.* (ISC); 19 Jun 1998, *Gilman et al. 98038* (VT); GNP, Round Prairie, 13 Jun 2003, *Carolin et al. s.n.* (ISC). New Mexico: Colfax Co., VPR, flat top of volcanic ridge, old logging road, 7 Aug 2008, *Legler 10425* (ISC, RM); 8 Aug 2008, *Legler 10426* (ISC, RM); VPR, Site 582, 23 Jul 2009, *Popovich & Farrar FL090723* (ISC); Colin Neblett State Wildlife Area, 30 Jul 2009, *Legler 11569* (RM). Taos Co., VPR, Costillo Creek, 27 Jun 2008, *Legler 9153* (ISC); Carson National Forest ("CNF"), Wheeler Peak Wilderness, 28 Jul 2009, *Legler 11554* (RM). Rio Arriba Co., Santa Fe National Forest, San Pedro Parks Wilderness, 5 Aug 2009, *Legler 11593* (RM); CNF, Cruces Basin Wilderness, 7 Aug 2009, *Legler 11616* (RM). South Dakota: Custer Co., Black Hills National Forest ("BHNF"), Cathedral Spires, 26 Jun 2011, *Mayer 1393* (ISC); BHNF, Hell Canyon, 5 Jun 2007, *Mergen 07B016A* (ISC). Lawrence Co., BHNF, Eagle Cliff, montane grassland, 19 Jun 2009, *Farrar 090619* (ISC); BHNF, Northern Hills, 24 Jun 2007, *Mergen 070524A* (ISC); BHNF, Riflepit Canyon, grassland, 15 Jun 2010 *Farrar FL100615* (ISC). Pennington Co., BHNF, Hat Mountain, 21 Jun 2011, *Farrar FL110621* (ISC); BHNF, Castle Peak, openings in ponderosa pine forest, 9 Jun 2007, *Mergen 07PC04C* (ISC); BHNF, Redbank Spring, 28 Jun 2006, *Farrar FL060628* (ISC);

BHNF, Reynolds Prairie, open prairie, 6 Jun 2009, *Mergen 09P006A* (ISC); BHNF, N of Moon, 5 Jun 2007, *Mergen 07B008A* (ISC). Wyoming: Big Horn: Bighorn NF ("BNF"), NE side Bald Mtn, 18 Aug 2010, *Legler 11813A* (RM, WTU). Crook Co., BHNF, Warren Peak, montane grassland, 1 Jun 2007, *Farrar FL070601* (ISC). Johnson Co., BNF, Forest Service Rd 31, 19 Aug 2010, *Popovich & Farrar FL100819* (ISC); BNF, Forest Service Rd 448 E of Munkres Pass, 18 Aug 2014, *Legler 13267* (ISC); BNF, Leigh Creek, 8 Aug 2012, *Legler 12473* (ISC); BNF, Pole Creek Rd, under roadside *Pinus contorta* saplings, 17 Aug 2009, *Legler 11631* (ISC); BNF, Powder River Pass, 22 Jul 2008, *Spann s.n.* (ISC). Sheridan Co., BNF, Forest Service Rd 26, 3 air-mi NW of Twin Lakes, 16 Aug 2010, *Legler 11805* (RM).

Supplemental data are available at the Farrar lab at Iowa State University or by contacting the authors, including additional specimens examined but not listed above, measurement data used in deriving the species description, and the geospatial data used for Fig. 8.

With the exception of *B.* "farrarii," an illustrated key that separates the below species from other *Botrychium* occurring within the known distributional range of *B. furculatum* is available (Farrar and Popovich, 2012).

KEY TO *B. FURCULATUM* AND SIMILAR SPECIES IN THE ROCKY MOUNTAIN REGION

1. Plants usually green when fresh; pinnae entire to symmetrically shallowly 3- or 5-lobed; stalks of lower pinnae appearing narrow, approximately 1/4 the pinna width; lower sporophore branches distinctly stalked, sporangia not obscuring the sporophore rachis . . . . *B. minganense*

1. Plants usually pallid, yellow- to white-green to glaucous blue-green when fresh; pinnae entire to crenate to asymmetrically cleft into two principal lobes, upper lobe often larger and more developed; stalks of lower pinnae not appearing narrow, approximately 1/3 or more the pinna width; lower sporophore branches usually short-stalked to sessile, sporangia partially obscuring the rachis 2

2. Plants glaucous blue-green; pinnae dome-shaped in outline, outer margins of pinnae (or if lobed, of pinnae lobes) entire; sporophore and trophophore stalks straight, joining in a straight-sided junction; spore size 31–39 μm (avg. 34) in longest diameter; plants of boreal North America and boreal areas in the Black Hills of South Dakota and Wyoming, not known from the Rocky Mountains . . . . *B. pallidum*

2. Plants glabrous to glaucous, yellow- to white-green, seldom glaucous blue-green; pinnae fan-shaped to spatulate in outline, sometimes ± rhombic, pinnae sometimes appearing stubby, truncate, or malformed, outer margins of pinnae and pinnae lobes often irregularly toothed or crenate; trophophore stalk often ± outwardly bowed, joining sporophore stalk in a spreading to narrow wishbone-like "V"; spore size 41–45 μm (avg. 43) in longest diameter; plants of the Rocky Mountains and the Black Hills . . . . *B. furculatum*

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A New Species of *Goniopteris* (Thelypteridaceae) from Hispaniola

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Abstract.—*Goniopteris baorucensis*, a species known only from the Sierra Baoruco in the southern Dominican Republic, is illustrated and described as new to science. Recent phylogenetic studies have inferred it as a member of a clade of predominantly calciphilic *Goniopteris* that are largely endemic to the Antilles. New records of two additional uncommon species in this clade, *G. hildae* and *G. nigricans*, are noted. A short discussion of the history of pteridology in Hispaniola is provided, with special reference to the contributions of Erik Ekman and Carl Christensen.

Resumen.—Se describe y ilustra *Goniopteris baorucensis*, una especie conocida solo desde la Sierra de Baoruco en el sur de la República Dominicana, como nueva para la ciencia. Estudios filogenéticos recientes lo han inferido como miembro de un clado de *Goniopteris* predominantemente calcícola que son en gran parte endémicos de las Antillas. Se observan nuevos registros de dos especies adicionales poco comunes en este clado, *G. hildae* y *G. nigricans*. También proporciono una breve discusión sobre la historia de la pteridología en La Española, con especial referencia a las contribuciones de Erik Ekman y Carl Christensen.

Key Words.—fern, Caribbean, Antilles, karst, calciphile, Ekman, Dominican Republic

Hispaniola is the second-largest island in the Caribbean, home to its highest mountains and greatest fern and lycophyte diversity, with 660 species estimated by Tryon (1979, 1986). Of these, some 45% are considered Antillean endemics, with 11% endemic to the island. The isolated mountain ranges and complex geology have likely contributed to diversification through both allopatry and edaphic specialization (Barrington, 1993; Smith, 1993). The greatest abundance and diversity of ferns in Hispaniola can be found in the tropical montane forest band, at middle to upper elevations, often bounded by pine-dominated forests (Martin, Sherman, and Fahey, 2007; Martin, Fahey, and Sherman, 2011). The intermontane valleys, especially in the rain shadows, are often dominated by dry forest vegetation, and host few ferns. Marine incursions into lowland valleys during the Pliocene and Pleistocene would have further contributed to the episodic isolation of these mountain ranges (Matos-Maraví *et al.*, 2014).

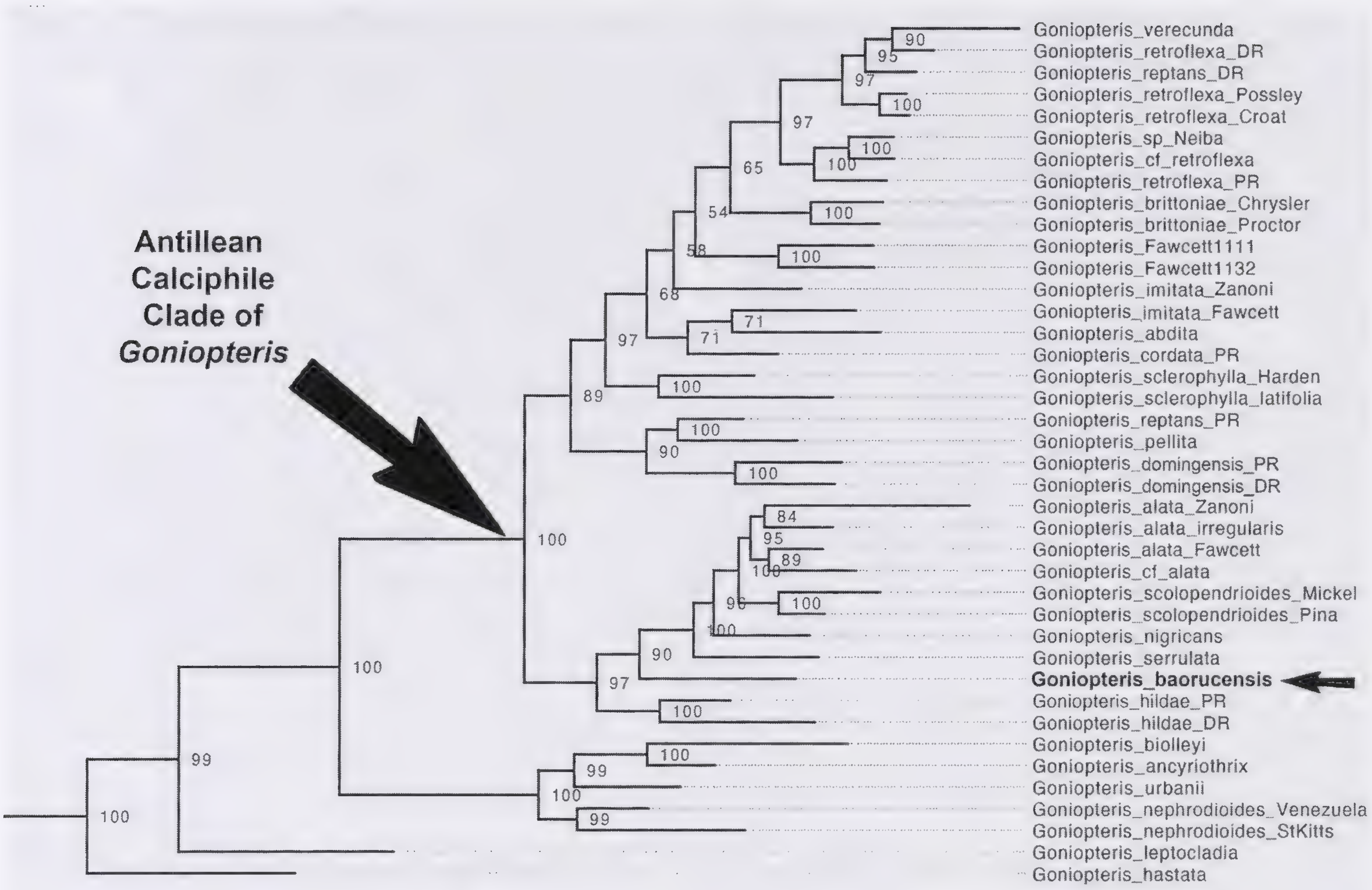
While recent decades have seen great advances in our knowledge of the fern and lycophyte flora of the other islands of the Greater Antilles (Proctor, 1985a, 1989; Sánchez, 2017), and the angiosperm flora of Hispaniola (Liogier, 1981), the fern and lycophyte diversity of Hispaniola remains comparatively little-known. Among the most prolific collectors of the Caribbean flora was Erik L. Ekman, who made more than 35,000 collections, resulting in the description of over 2000 species new to science. Among these, 2000 collections of ferns and lycophytes from Hispaniola were made between 1917 and 1930; these represent more than 600 species (Christensen, 1937). Ekman's spermatophyte collections were sent to Otto Urban (B) for determination, while the ferns and lycophytes (excluding *Selaginella*) were sent to Carl Christensen (C), who frequently collaborated with William Maxon (US) on species descriptions. The study of Ekman's collections ultimately resulted in the publication of 43 new species of ferns and lycophytes, including four new species of *Goniopteris* (Christensen, 1937). Few mountain ranges within Cuba, Haiti, or the Dominican Republic were left unexplored by Ekman. The Dominican province of Barahona is noteworthy as one of the few places he barely explored, though he had in fact planned a collecting trip there (Christensen, 1937) just before "he died suddenly from a combined attack of malaria, black water fever, and pneumonia on Jan. 15, 1931" at the age of 48 (Howard, 1952:83).

Through the extraordinary collecting efforts of Ekman and the insightfulness of Christensen (especially for members of the Thelypteridaceae), we have a greatly improved understanding of Hispaniolan fern diversity, though more work remains to be done. In addition to Ekman's work, the efforts of other general collectors, notably Thomas Zanoni (NY), Walter Judd (FLAS), and Teodoro Clase (JBSD), have contributed many additional fern specimens, but few fern and lycophyte specialist collectors have focused their attention on Hispaniola (but see Howard, 1950; Leonard, 1925a, b; Mickel, 1997).

With the goal of documenting fern and lycophyte diversity, especially targeting regions not well explored by Ekman, a collecting trip to the Dominican Republic was organized in December 2018. Expedition members included Teodoro Clase (JBSD), Weston Testo (FLAS), Lindsey Riibe (FLAS), Sally Chambers (SEL), Pedro Schwartsburd (VIC), Yommi Piña (JBSD), Yuley Piñeyro (JBSD), and myself (VT).

The Antillean Calciphile Clade of *Goniopteris*

The genus *Goniopteris* includes approximately 120 species (PPG I, 2016) and is widespread throughout the mid and lower elevations of the Neotropics (Fawcett and Smith, in press). Smith (1993), in his discussion of the role of edaphic specialization in fern biogeography, recognized a group of species in *Goniopteris* that exemplify the phenomenon; these species are united by several morphological traits and characteristically prefer rocky, calcareous substrates. He believed them to constitute a clade, an insight that is well-supported by molecular evidence (Fawcett *et al.* in review; Fig. 1). This group of Antillean calciphiles, which includes about 30 minimum rank taxa, shows extremely high rates of endemism, with only a few of its species (*e.g.*, *G. domingensis* (Spreng.) Pic. Serm., *G. pellita* (Willd.) A.R.Sm., *G. reptans* (J.F.Gmel.) C.Presl, and *G. sclerophylla* (Poepp. ex Spreng.) Wherry) having secondarily dispersed to continental areas bordering the Caribbean (Lange and Angelo, 2020; Mickel and Smith, 2004; Morton, 1951). Several species are narrowly endemic to a single mountain range (Caluff and Sánchez, 2004), and five of the ten members of this clade in Puerto Rico were considered single-


Antillean Calciphile Clade of *Goniopteris*
Goniopteris_verecunda
Goniopteris_retroflexa_DR
Goniopteris_reptans_DR
Goniopteris_retroflexa_Possley
Goniopteris_retroflexa_Croat
Goniopteris_sp_Neiba
Goniopteris_cf_retroflexa
Goniopteris_retroflexa_PR
Goniopteris_brittoniae_Chrysler
Goniopteris_brittoniae_Proctor
Goniopteris_Fawcett1111
Goniopteris_Fawcett1132
Goniopteris_imitata_Zanoni
Goniopteris_imitata_Fawcett
Goniopteris_abdita
Goniopteris_cordata_PR
Goniopteris_sclerophylla_Harden
Goniopteris_sclerophylla_latifolia
Goniopteris_reptans_PR
Goniopteris_pellita
Goniopteris_domingensis_PR
Goniopteris_domingensis_DR
Goniopteris_alata_Zanoni
Goniopteris_alata_irregularis
Goniopteris_alata_Fawcett
Goniopteris_cf_alata
Goniopteris_scolopendrioides_Mickel
Goniopteris_scolopendrioides_Pina
Goniopteris_nigricans
Goniopteris_serrulata
Goniopteris_baorucensis
Goniopteris_hildae_PR
Goniopteris_hildae_DR
Goniopteris_biolleyi
Goniopteris_ancyriothrix
Goniopteris_urbanii
Goniopteris_nephrodioides_Venezuela
Goniopteris_nephrodioides_StKitts
Goniopteris_leptocladia
Goniopteris_hastata
90
95
97
100
97
100
100
65
54
100
58
100
68
71
71
97
100
89
100
90
100
84
95
89
100
96
100
100
90
97
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100
99
100
99
99
100

FIG. 1. Maximum likelihood tree inferred from a concatenated matrix of 407 individual gene trees. The clade of interest, demonstrating the position of *Goniopteris baorucensis* and relationships among the Antillean calciphile clade was pruned from the original 621 accession tree of Fawcett *et al.* (in review). The branches are annotated with ultrafast bootstrap support values (Hoang *et al.*, 2017).

island endemics by Proctor (1989). Another feature of these species is their great propensity for hybridization, with more than a dozen putative hybrids among members of the genus in Cuba proposed by Sánchez (2017), and many herbarium collections exhibiting intermediate morphology and the misshapen spores and sporangia often indicative of F_1 hybrids (Wagner, Wagner, and Taylor, 1986). Several described species have been hypothesized to be hybrids, or of hybrid origin (*e.g.*, *G. dissimulans* (Maxon & C.Chr.) Salino & T.E.Almeida, Christensen, 1913; *G. verecunda* (Proctor) Salino & T.E.Almeida, Proctor, 1985b; 1989), or have been described as intermediate between other taxa (*e.g.*, *G. subsagittata* (Maxon & C.Chr.) Salino & T.E.Almeida; Christensen, 1937). Limited cytological work has revealed that certain taxa are represented by both diploid and tetraploid cytotypes, *e.g.*, *G. reptans* (Smith, 1971; Wagner, 1963; Walker, 1966) and *G. retroflexa* (L.) Salino & T.E.Almeida (Walker, 1966), signaling potential cryptic speciation (Barrington, Haufler, and Werth, 1989).

A recent phylogenomic study of the Thelypteridaceae (Fawcett *et al.*, in review) included 32 accessions from the Antillean calciphile clade of *Goniopteris* (Fig. 1), representing 17 taxa, including the new species described herein. The maximum likelihood phylogeny was inferred from a 407 nuclear locus target-enriched dataset using the *GoFlag 408* probe set (Breinholt *et al.*, 2020). The crown node of this clade is well-supported, as are many other clades (including several of those with more than one accession of the same species), but backbone support is mixed. Recent rapid radiation, hybridization, reticulation, and polyploidy, in addition to gene tree estimation error, may explain remaining uncertainty about some of these relationships (Degnan and Rosenberg, 2009). A subset of the 621 accession Thelypteridaceae tree was pruned to show inferred relationships among the Antillean calciphile clade. *Goniopteris serrulata* (Sw.) J.Sm., *G. hildae* (Proctor) Salino & T.E.Almeida, and *G. alata* (L.) Ching exhibit diverse and distinctive morphologies (Fig. 2), but are resolved as closely related to *G. baorucensis* (Fig. 1). The concatenated maximum likelihood tree annotated with gene and site concordance factors (Minh *et al.,* 2020), a multispecies coalescent tree (Zhang *et al.,* 2017), and the sequence alignment are available on Dryad doi.org/10.5061/dryad.gxd2547j4, and were included in Fawcett *et al.* (in review).

Taxonomic Treatment

Goniopteris baorucensis S.E.Fawc. **sp. nov.**

Type: DOMINICAN REPUBLIC. Barahona: Camino de la Hoz (dirt road to summit of Monte La Jo), 18.17497, -71.28118, cloud forest remnant, on steep, shaded roadside bank, 1438 m, December 2018, *Fawcett 1068* (holotype: VT; isotypes: UC, JBSD).

Description

Plants terrestrial, **rhizomes** short-creeping to erect, 1–2 cm wide, to 6 cm tall; **fronds** erect, arching or pendant, to 70 cm long, 8 cm wide; **stipes** stramineous to dull brown when dried (stipe bases purplish when fresh), densely stellate-puberulent, 1–2 mm wide, typically 1/3–2/5 of total mature frond length; **stipe scales** basifixed, drying castaneous (purplish when fresh), tapering, linear-lanceolate, with stellate hairs on margins and surfaces, to 1 mm wide and 1 cm long; **blades** narrowly elliptic, pinnate, chartaceous, in deep shade dark bluish green-iridescent adaxially, pale green abaxially, laminae paler in sunnier sites, drying dull dark green to gunmetal gray; **pinnae** opposite, subopposite, or alternate, in 20–30+ free pairs before becoming adnate into gradually attenuate frond apex, each pinna 3–7 mm wide, 18–45 mm long, spreading to falcate, margins entire (rarely subentire), revolute in sunnier sites, apices broadly acute to rounded, bases adnate to sessile, or a few proximal pinna pairs short-petiolulate with truncate to shallowly cordate bases, sometimes with slightly expanded, rounded acroscopic and basiscopic auricles; **veins** free and simple in smallest pinnae, forked or with one vein-pair anastomosing in largest pinnae; **indument** of short, stellate hairs, these short-stalked with (4–) 5–7 (–8) branches, present on rhizome and stipe scales, stipes, rachises, abaxial costae, and indusia, such hairs lacking or fugacious on costae adaxially, scattered on abaxial secondary veins and laminae, simple hairs lacking throughout; **sori**

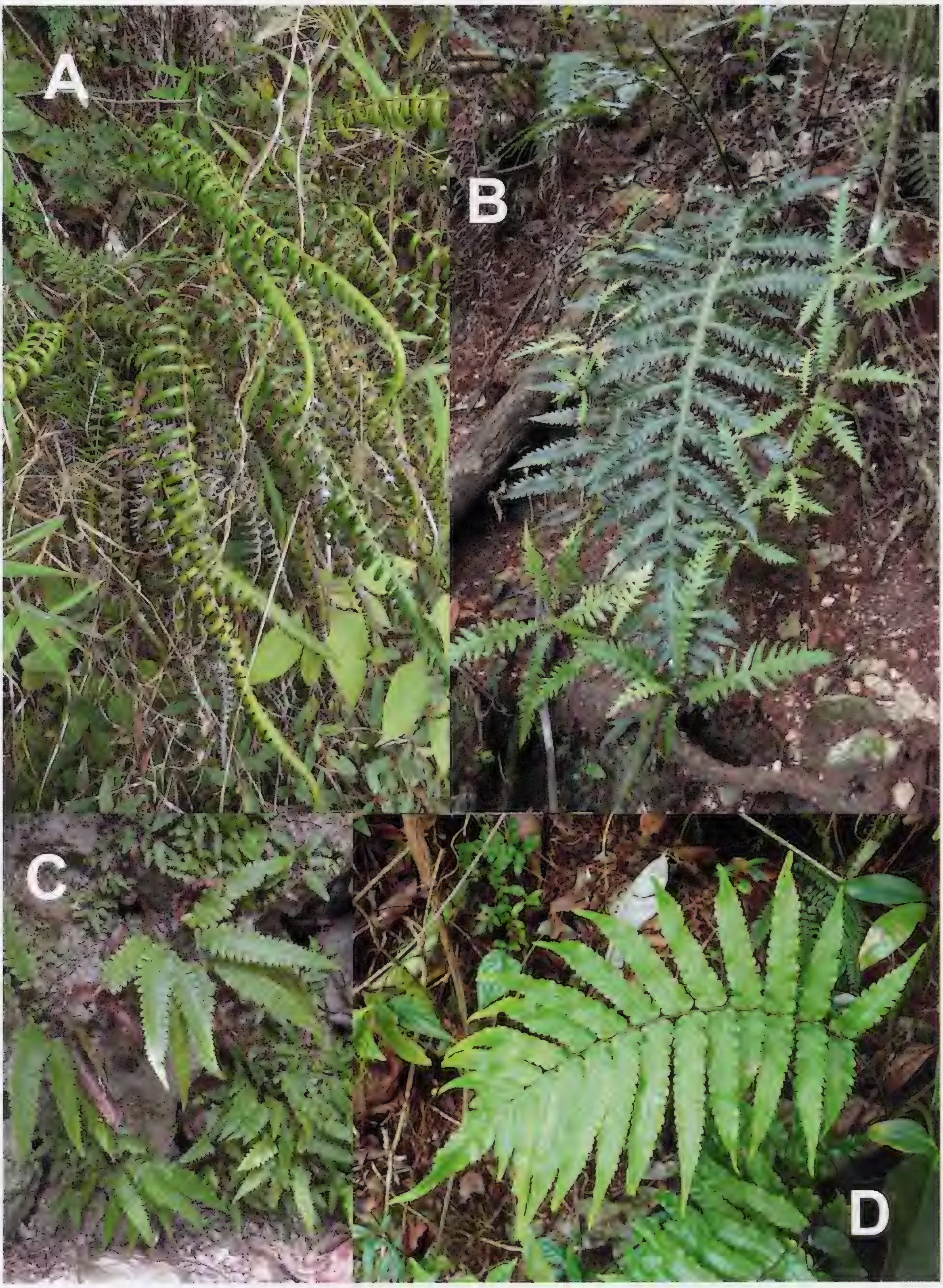

FIG. 2. Growth habit of *Goniopteris baorucensis* and three closely related species. A. *Goniopteris baorucensis*, Dominican Republic: Barahona, *Fawcett 1068* (VT). B. *Goniopteris alata*, Dominican Republic: Pedernales, *Fawcett 1077* (VT); C. *Goniopteris hildae*, Puerto Rico: Hatillo, *Fawcett 400* (VT), D. *Goniopteris serrulata* Jamaica: Trelawny, *Fawcett 1256* (IJ).

round, discrete, medial, irregularly uniseriate to biseriate, borne singly or paired on forked veins; **indusia** large, thick, persistent, brown, with dense, pale stellate hairs on surfaces and margins; **sporangia** glabrous; **spores** light brown, monolete (Figs. 2A, 3).

PARATYPES: DOMINICAN REPUBLIC. Barahona: Camino de la Hoz (dirt road to summit of Monte La Jo), 18.17497, -71.28118, cloud forest remnant, on steep, shaded roadside bank, 1438 m, 6 December 2018, *Fawcett 1069, 1070, 1071* (UC).

DIAGNOSIS

Goniopteris baorucensis is readily distinguished from all other members of the genus by its long, once-pinnate, narrowly elliptic fronds (length to width ratio 8–12), and widely spaced, elongate, entire pinnae (length to width ratio (4–) 6–10 (–13)). *Goniopteris baorocuensis* is superficially similar to *G. retroflexa* and *G. imitata*, two species that occur in proximity, and in similar habitats on Hispaniola. *Goniopteris retroflexa* differs in the presence of long simple hairs intermixed with short-stellate hairs, with petiolulate, occasionally hastate pinnae (vs. most pinnae adnate to sessile, proximal pinnae petiolulate, with slightly expanded, rounded auricles) and pinna margins typically lobed to crenate (vs. (sub)entire). *Goniopteris imitata* (C.Chr.) Salino & T.E.Almeida differs in its smaller stature, usually less than 40 cm (vs. up to 70 cm), with medial pinnae petiolulate (vs. adnate to sessile), frond apex short-attenuate (vs. frond apex long-attenuate), indusia gray (vs. indusia brown), and stellate hairs with mostly 2–4 branches (vs. 4–8 branches). Among closely related congeners (Figs. 1, 2), *G. baorucensis* is unique in having once-pinnate laminae with entire pinnae; *G. scolopendrioides, G. hildae* (Fig. 2C), and *G. alata* (formas *typica* (Fig. 2B), and *subbipinnata*) have at most a few free pinna pairs proximally; *G. nigricans, G. alata* var. *irregularis*, and *G. serrulata* (Fig. 2D) are pinnate-pinnatifid. The Jamaican endemic *Goniopteris serrulata* is further distinguished by its large size, with erect caudices that may be 10 cm thick and 45 cm tall.

PUTATIVE HYBRIDS

As for many other members of the Antillean calciphile clade of *Goniopteris*, hybrids involving *G. baorucensis* are suspected on the basis of intermediate morphology. *Goniopteris retroflexa* (syn.: *Thelypteris asplenioides* (Sw.) Proctor) is one of the most widespread and abundant species, occurring on all four islands of the Greater Antilles. This species and *G. sclerophylla* share the calcareous montane cloud forest habitat at the type locality of *G. baorucensis*. Individuals intermediate between *G. baorucensis* and *G. retroflexa, e.g., Fawcett 1054* (UVMVT291536) and *Fawcett 1041C* (UC), bear both stellate and simple hairs, and have petiolulate medial pinnae with hastate auricles, undulate margins, and fugacious indusia. However, these plants exhibit habit and architecture (long narrow pinnae and leaf blades) similar to

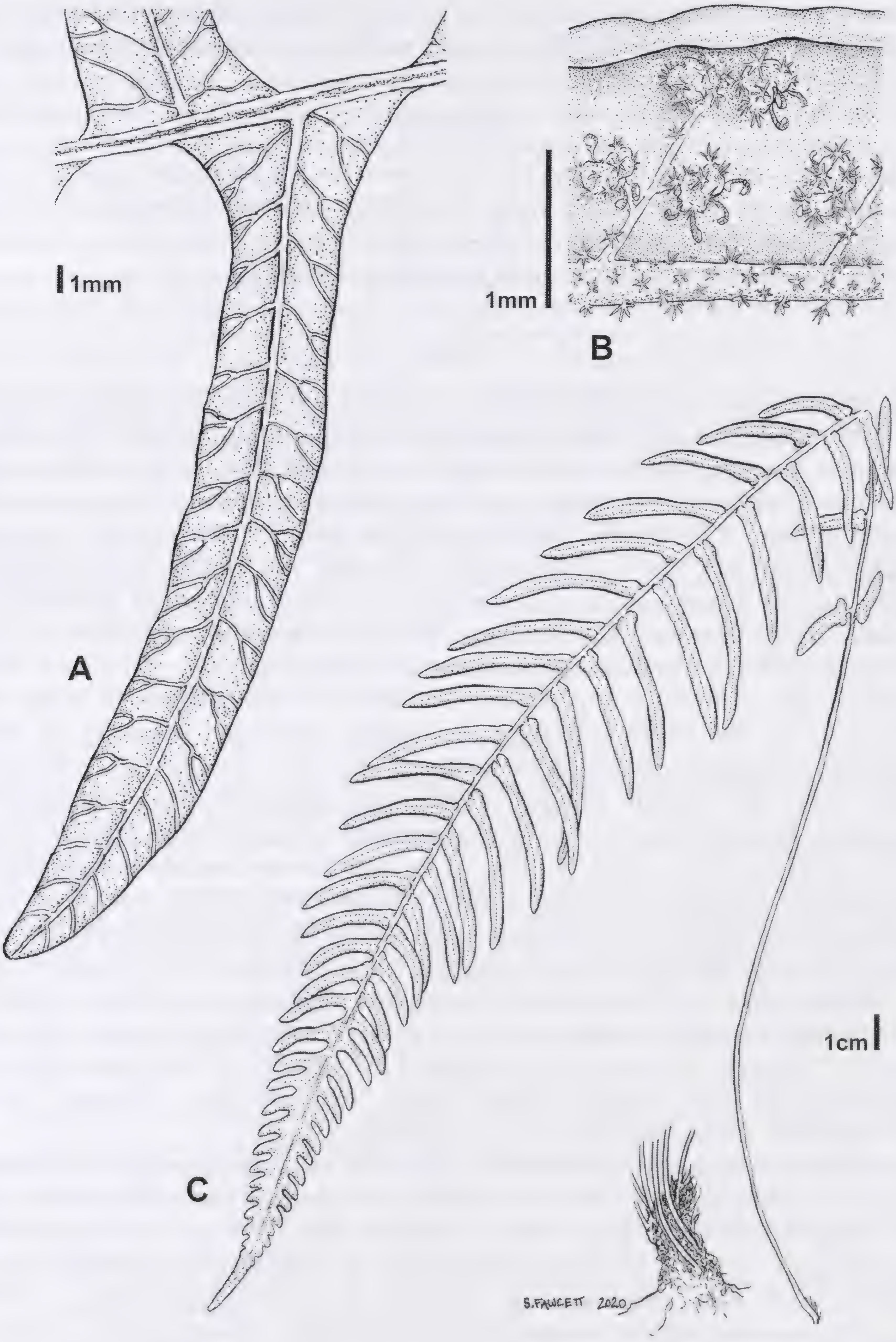

FIG. 3. *Goniopteris baorucensis.* A. Adaxial view of medial pinna, *Fawcett 1068* (UC); B. Abaxial view of medial pinna, *Fawcett 1068* (UC) C. Frond and rhizome, *Fawcett 1071* (UC).

G. baorucensis. Additional collections from the Massif de la Selle to the west in Haiti (*e.g.*, *Zanoni 18679*, NY, image!) resemble these putative hybrids.

Etymology

Named for the type locality, in the Sierra de Baoruco, the southernmost mountain range of the Dominican Republic. Baoruco (alternately spelled Bahoruco) has been interpreted as a toponym of Macorís derivation, meaning "within the jungle" (Granberry and Vescelius, 2004).

Notes

Based on both morphological (Figs. 2, 3) and phylogenetic criteria (Fig. 1), *Goniopteris baorucensis* warrants recognition as a distinct species. It is known only from the type locality, the summit area of Monte La Jo, a remnant of montane cloud forest over limestone, fragmented by pasture. It occurs in the understory and at the edges of these broadleaf forests. It may occur in similar habitats along the Sierra Baoruco and Tiburon Peninsula, and should be sought on the Massif de la Selle, which is a continuation of the range in southern Haiti. Because of its rarity, narrow endemism, and threats to its habitat, it is of considerable conservation concern.

During the collecting expedition, we had an opportunity to study the collections at the JBSD herbarium in Santo Domingo, resulting in some noteworthy findings. *Goniopteris hildae* (Fig. 2C), considered endemic to Puerto Rico by Proctor (1985b, 1989), was collected from northeastern Dominican Republic, in the province of Samaná (*Zanoni 35382*, JBSD!; *Zanoni 17651*, JBSD!; *Mejia 1619*, JBSD!). The specimens had been previously annotated as *G. hildae* by Alan Smith (UC) in 1996. Mejia's specimen from the Dominican Republic resolves as sister to the Puerto Rican accession of *G. hildae* (*Fawcett 369*, VT) in the maximum likelihood analysis of Fawcett *et al.* (in review) (Fig. 1). *Goniopteris nigricans* (Ekman & C.Chr.) Salino & T.E.Almeida, heretofore apparently known only from the type material (*Ekman 10146*, NY image!, US image!) from Massif de la Hotte in Haiti, was collected there again in 2006 (*Hilaire* 2405, JBSD!), and was also included in the phylogenomic study (Fawcett *et al.*, in review; Fig. 1).

Lastly, there has been considerable confusion as to the appropriate name for the species most recently known as *Goniopteris moranii* (Sánchez, 2017; Lange and Angelo, 2020). This name is based on the type of *Polypodium guadalupense* Wikstr., but the combination in *Goniopteris* is blocked by *G. guadalupensis* Fée, considered a synonym of *G. tetragona* (Sw.) C. Presl. However, several earlier synonyms are available, rendering the name *G. moranii* superfluous. The earlier name *Polypodium scolopendrioides* L. has often been considered a synonym *of P. guadalupense* Wikstr., but study of the type material, *i.e.,* plate 91 of Plumier (1705) cited by Linnaeus (1753: 1085) and so lectotypified by Christensen (1913: 212), and the associated epitype (*Tournefort 5292*, P00322174, image!; designated by Cremers & Aupic 2008), confirm that these two species are related, but distinct taxa, as realized by Christensen (1913) and others. The oldest available synonym is *Polypodium domingense* Spreng., and thus the appropriate name for this widespread Caribbean taxon is *Goniopteris domingensis* (Spreng.) Pic. Serm. For detailed synonymy and additional nomenclatural discussion, see Christensen (1913).

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There and Back Again: Reticulate Evolution in *Ceratopteris*

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Abstract.—Examples of reticulate evolution are known from throughout the tree of life, but are particularly common in ferns due to their unique reproductive biology and few prezygotic reproductive barriers. However, untangling the complex evolution history of these groups can be challenging. Often, several different types of data are needed to understand the full story of reticulate evolution; for example, chloroplast markers trace maternal inheritance, while nuclear markers complete the picture by suggesting paternal inheritance. Next generation sequencing can provide thousands of nuclear loci, which are informative for estimating reticulate evolutionary histories. The model fern genus *Ceratopteris* is known to have cryptic allotetraploid taxa, and hybridization is common between many species in the genus. To better understand the patterns of hybridization and reticulate evolution in the genus, we constructed a split network analysis using thousands of single nucleotide polymorphisms from samples collected throughout the pan-tropical range of *Ceratopteris*. Our split network organizes taxa based on genomic similarity, revealing potential introgression between lineages. Combining this analysis with Patterson's D to measure gene flow, as well as contributions by previous authors, we show extensive hybridization and reticulate evolution in Old World *Ceratopteris*, and also provide evidence for natural hybridization events involving the model species *C. richardii*.

Key Words.—polyploidy, hybridization, RADseq, network analysis, cryptic species

Phylogenetic relationships are often depicted with neatly bifurcating trees, but such phylogenies do not always portray the full picture of evolution, especially within closely related groups (Otto and Whitton, 2000; Stebbins, 1950). When estimating the topology of an evolutionary tree, one should consider not just the steady accumulation of lineages through divergent evolution, but also the rejoining of branches via reticulate evolution. Reticulate species complexes are known from many lineages including animals (Hebert *et al.*, 2004; Nygren, 2014), fungi (del Carmen Molina *et al.*, 2011), and angiosperms (Ma *et al.*, 2018; Nauheimer *et al.*, 2019). Species complexes are particularly common in ferns, because spore-dispersed plants lack the prezygotic barriers created by intricate floral morphology of many angiosperms (Ranker and Sundue, 2015; Rothfels *et al.*, 2015; Smith, 1972), leading to high rates of hybridization and polyploidy (Barrington, Haufler and Werth, 1989; Sigel, 2016). This lack of prezygotic barriers in ferns allows hybridization to occur even across deep divergences (*e.g.*, 60 Mya in ×*Cystocarpium* [Rothfels *et al.*, 2015]; 50 Mya in ×*Lindsaeosoria* [Lehtonen, 2018]; 40 Mya in *Dryopteris* [Sessa, Zimmer, and Givnish, 2012a]). Hybridization is often followed by polyploidization, which can occur either via a triploid bridge (Gastony, 1986; Yamauchi *et al.*, 2004), or genome-doubling event (Glover *et al.*, 2016; Soltis and Soltis, 2009). Polyploidy also helps to mask genetic load (Haufler *et al.*, 2016) and reduce inbreeding depression (Masuyama and Watano, 1990).

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* Corresponding author

While prevalent in ferns, reticulate evolution can be difficult to discern. Historically, a combination of morphological, cytological, chloroplast (plastid), nuclear data, and experimental crossing tests was required to disentangle reticulate hybrid complexes (Barrington, Haufler, and Werth, 1989; Masuyama *et al.*, 2002; Masuyama and Watano, 2010; Sessa, Zimmer, and Givnish, 2012b). Such work was time-consuming and laborious, because detecting and parsing the complex signals of historic, reticulate evolution requires careful data curation (*e.g.*, Linder and Rieseberg, 2004; Masuyama, 2008; Masuyama *et al.*, 2002; Masuyama and Watano, 2005). The advent of next generation sequencing has created techniques that can generate up to tens of thousands of loci to test hypotheses about reticulate evolution. In particular, restriction site-associated DNA sequencing (RADseq) is a cost-effective way to generate these data (Blanco-Pastor *et al.*, 2019; Eaton and Ree, 2013; Ma *et al.*, 2018; Moura *et al.*, 2020). Such approaches are revolutionizing the study of reticulate evolution in other taxa, and also have the potential to do so for ferns (Dauphin *et al.* 2017; Wolf *et al.*, 2019; Kinosian *et al.*, 2019; Kinosian, Pearse, and Wolf, 2020).

The fern genus *Ceratopteris* is best known for its utility as a model organism. The species *Ceratopteris richardii* Brong. was the first homosporous fern to have a partial whole genome sequence available (Marchant *et al.*, 2019), and has been important to research topics such as sex determination (Banks, 1997), leaf development (Vasco *et al.*, 2016), and apogamy (Bui *et al.*, 2017). In addition to its contributions as a model organism and genomic resource, *Ceratopteris* provides an excellent system for the study of reticulate evolution. The genus consists of three diploid and five tetraploid species (see Table 1; Lloyd, 1974; Masuyama and Watano, 2005; Zhang *et al.*, 2020). Many of these are hypothesized to be of hybrid origin (Adjie *et al.*, 2007; Lloyd, 1974), with potential backcrossing between some lineages (Hickok and Klekowski, 1974). Although there are studies of reticulate evolution in Old World *Ceratopteris* (Adjie *et al.*, 2007), such relationships in other areas of its pan-tropical range have not been examined thoroughly.

Here, we present a network analysis of the species relationships within *Ceratopteris* using a multi-locus nuclear dataset. This information, along with estimations of gene flow, previously published gene trees, and population genomic analyses, enable us to understand reticulate evolution in this genus to a finer degree. We present evidence for extensive, ancient hybridization and reticulate evolution in both Old and New World *Ceratopteris*. In addition, our data suggest natural hybridization events involving the model species *C. richardii* (discussed in Hickok and Klekowski, 1974; Nakazato *et al.*, 2007). Although hybridization and reticulations were suspected in *Ceratopteris,* our network analysis shows how important these events have been throughout the evolutionary history of the genus.

MATERIALS AND METHODS

Our sampling, DNA extraction and sequencing, and data processing (but *not* data analysis) are described in detail in (Kinosian, Pearse, and Wolf, 2020); however, we give an overview here. A detailed description of all steps and parameters of our data processing pipeline can be found on GitHub (github.com/sylviakinosian/ceratopteris_RADseq). Demultiplexed sequences can be accessed via the NCBI GenBank Short Read Archive (PRJNA606596).

Sampling.—To obtain samples from throughout the pan-tropical range of *Ceratopteris,* we used a combination of herbarium and silica-dried fresh samples. We collected a total of 90 samples, 56 from herbarium specimens in the U.S. and 34 from the field in Taiwan, China, Costa Rica, and Australia. These samples represent five of the eight named species of *Ceratopteris* (see Table 1 for species descriptions), and one sample of *Acrostichum aureum* L. from Australia for use as an outgroup. Full specimen collection data are given in the supplementary materials (Online Supplementary Data 1).

We identified specimens in the field by consulting the dichotomous keys and illustrations in Lloyd (1974) and Masuyama and Watano (2010). For herbarium specimens, we used the name given by the collector, or the most recent annotation. If a specimen was identified as a different species by our genomic analyses, we utilized the above keys to double check this identification with the digitized specimen or photographs taken by SPK during material collection.

DNA extraction and sequencing.—Most samples were supplied as silica-dried leaf tissue to the University of Wisconsin-Madison Biotechnology Center, where DNA was extracted using the QIAGEN DNeasy mericon 96 QIAcube HT Kit. The remainder (17 samples) were extracted from silica-dried leaf tissue using a modified CTAB method by SPK at Utah State University. Both the University of Wisconsin-Madison Biotechnology Center and SPK used variants of the CTAB DNA extraction method (Doyle and Doyle, 1987), as research suggests it is the best for extracting high-purity DNA from herbarium specimens (Särkinen *et al.*, 2012). All specimens were sent to the University of Wisconsin-Madison Biotechnology Center, analyzed for quality, and then pooled with the rest of the samples. Restriction site-associated DNA sequencing (RADseq) libraries were prepared by University of Wisconsin-Madison Biotechnology Center, following Elshire *et al.* (2011) with minimal modification; sequencing was performed on Illumina NovaSeq 6000 2x150 S2.

TABLE 1. Named species of *Ceratopteris*. There are currently three diploid and five tetraploid species with published names; additional cryptic variation is suspected in *C. thalictroides* and *C. cornuta*. The authority, ploidy, spores per sporangium, range, potential hybridization, and relevant citations are listed.

Species name	Authority	Ploidy, spores	Geographic range	Evidence of hybridization	Notes	Citations
Ceratopteris richardii	Brong.	diploid ($n = 39$), 16 spores per sporangium	Central America, Western Africa	Yes, with *C. pteridoides* and *C. thalictroides* (PN8)	Model species; deltoid lamina	Lloyd (1974), Hickok and Klewkowski (1974), Nakazato *et al.*, 2007)
C. pteridoides	(Hook.) Hieron	diploid ($n = 39$), 32 spores per sporangium	Southern North America (Gulf of Mexico coast), Central America, northern South American	Yes, with *C. richardii*	Plants often floating; inflated stipes; distinct genomic population	Lloyd (1974), Hickok and Klewkowski (1974); Kinosian, Pearse and Wolf (2020)
C. cornuta	(Pal. Beauv.) Le Prieur	diploid ($n = 39$), 32 spores per sporangium	Sub-Saharan Africa, Arabian Peninsula, India, SE Asia, N. Australia	Hypothesized progenitor of *C. gaudichadii* and *C. oblongiloba*	Plants rooted or floating; highly gemmiferous	Lloyd (1974), Adjie *et al.* (2007)
C. thalictroides	(L.) Brong.	tetraploid ($n = 77$), 32 spores per sporangium	India, SE Asia, Australia, Japan, China, Taiwan, Central America	Yes, with *C. gaudichaudii* and *C. richardii* (PN8)	Highly polymorphic; Central American population may be separate species	Lloyd (1974); Nakazato *et al.*, 2007); Masuyama and Watano (2010); Kinosian, Pearse, and Wolf (2020)
C. gaudichaudii var. *gaudichaudii*	Brong.	tetraploid ($n = 78$), 32 spores per sporangium	Endemic to Guam	unknown	Cryptic sp. of *C. thalictroides*; larger variety; highly gemmiferous	Masuyama and Watano (2010)

Table 1. Continued.

Species name	Authority	Ploidy, spores	Geographic range	Evidence of hybridization	Notes	Citations
C. gaudichaudii var. *vulgaris*	Brong. (Masuyama & Watano)	tetraploid ($n = 78$), 32 spores per sporangium	China, Japan, Nepal, Hawaii, Guam, N. Australia	Yes, with *C. oblongiloba* and *C. thalictroides*	Cryptic sp. of *C. thalictroides*; smaller variety, more common	Masuyama and Watano (2005); Masuyama and Watano (2010)
C. oblongiloba	Masuyama & Watano	tetraploid ($n = 78$), 32 spores per sporangium	Indonesia, Philippines, Thailand	Yes, with *C. gaudichaudii*	Cryptic sp. of *C. thalictroides*; highly dissected	Masuyama and Watano (2005); Masuyama and Watano (2010)
C. froesii	Brade	Unknown, probably tetraploid	Endemic to Brazil	unknown	Very small plants, <3cm tall; considered dwarf *C. thalictroides* by Lloyd	Lloyd (1974), Masuyama and Watano (2010)
C. shingii	Y. H. Yan & R. Zhang	tetraploid ($n = 77$)	Hainan Province, China	unknown	Cryptic sp. of *C. thalictroides*; plants with creeping rhizomes; grows on volcanic rock	Zhang *et al.* (2020)

Data processing.—RADseq yields millions of sequences that need to be filtered and processed before data analysis. Raw data were demultiplexed using *stacks* v. 2.4 *process_radtags* (Catchen *et al.*, 2013; Catchen *et al.*, 2011). The resulting demultiplexed FASTQ files were paired, and low quality bases, adapters, and primers removed using *ipyrad* version 0.9.52 (Eaton and Overcast, 2020). We used default settings for the remainder of data processing, with the exception that we required a locus to have a sequencing depth of at least 6, and data from a minimum of 30 samples for inclusion in the final assembly to reduce missing data, due to the unbalanced nature of our sampling (discussed in Eaton *et al.*, 2017; Hou *et al.*, 2016).

For the purpose of this study we use the *ipyrad* pipeline's definition of a locus as a short sequence present across samples. Single nucleotide polymorphisms (SNPs) are selected from each locus and used in downstream analyses. Our initial sampling contained 90 unique specimens, plus six replicate samples taken randomly from the 90 unique samples. Samples that were low quality (yielding <1000 loci) were removed from downstream analysis to limit missing data. Our final data set included 50 samples of *Ceratopteris* and one sample of *Acrostichum aureum*. No specimens identified by their collectors as the model species *C. richardii* made it through our data processing due to low quality and sequence coverage. However, we believe that some specimens identified as other taxa were misidentified, and potentially could be *C. richardii.*

Estimation of interspecies gene flow.—We calculated Patterson's D (also known as the ABBA-BABA or D-statistic) using the program *Dsuite* (Malinsky, Matschiner and Svardal, 2020). This statistic measures divergence from a bifurcating evolutionary history (see Green *et al.*, 2010; Durand *et al.*, 2011). *Dsuite* uses biallelic SNPs to estimate gene flow within species quartets, including three ingroup and one outgroup taxa; a D-statistic is reported for each quartet. We used the clades that were identified by Kinosian, Pearse, and Wolf (2020): *C. pteridoides*, *C. gaudichaudii*, *C. cornuta*, Old World *C. thalictroides*, and New World *C. thalictroides*, with *Acrostichum aureum* as the outgroup in all quartets.

Phylogenetic network analysis.—Huson and Bryant (2006) define three types of phylogenetic networks: traditional phylogenetic trees, split networks, and reticulate neworks. Split networks are *implicit* representations of evolution, and can depict multiple phylogenetic hypotheses in one figure, as well as incongruities between them. Reticulate networks, unlike split networks, are *explicit* evolutionary reconstructions and so their internal nodes represent ancestral individuals (Huson and Bryant, 2006). Split networks are a good way to explore data that may not be suitable for traditional phylogenetic approaches, such as a group known to have a reticulate evolutionary history.

To investigate the complex evolutionary history of *Ceratopteris*, we utilized the NeighborNet (Bryant and Moulton, 2002) split network algorithm in SplitsTree v. 4.16.1 (Huson and Bryant, 2006; Fig. 1). NeighborNet is similar to a neighbor joining (NJ) tree in that it pairs samples based on similarity, agglomerating taxa into larger and larger groups (Bryant and Moulton, 2002).

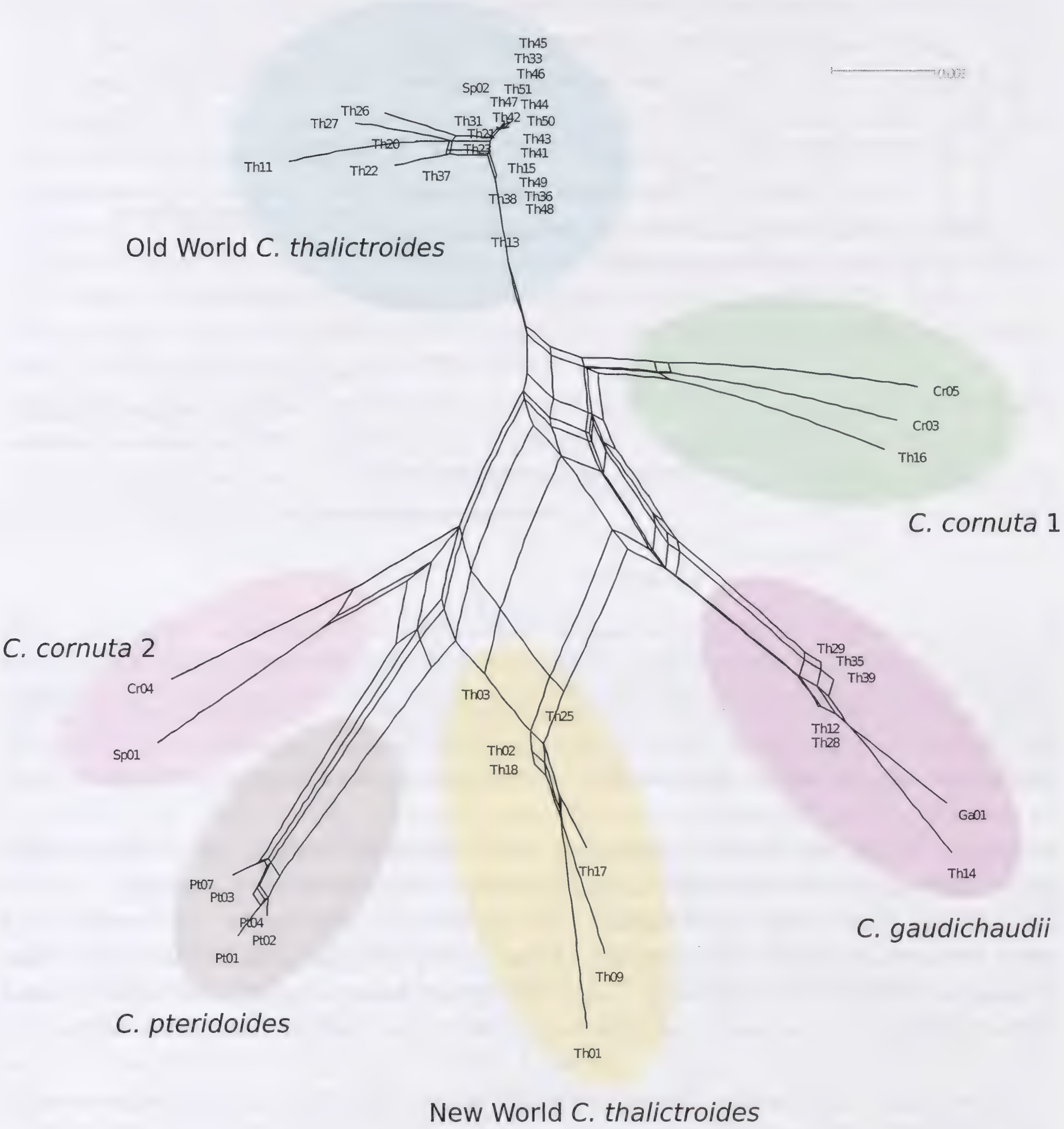

FIG. 1. Network analysis of *Ceratopteris* using SplitsTree4. Branch tips represent individuals, and each major group is labeled by species. Groups that share more links/sections of the network are hypothesized to have reticulate gene flow.

Unlike NJ, however, NeighborNet does not produce a bifurcating tree: it creates a split network, where parallel lines indicate splits of taxa, and boxes created by these lines indicate conflicting signals (Bryant and Moulton, 2002). The handful of phylogenetic studies on *Ceratopteris* have yielded slightly different topologies (Adjie *et al.*, 2007; Kinosian, Pearse, and Wolf, 2020; Zhang *et al.*, 2020), so we wanted to explore a method outside of a bifurcating tree to better understand the evolutionary history of the group. In addition, we conducted a Phi test (Bruen, Philippe, and Bryant, 2006) using SplitsTree. This test is used

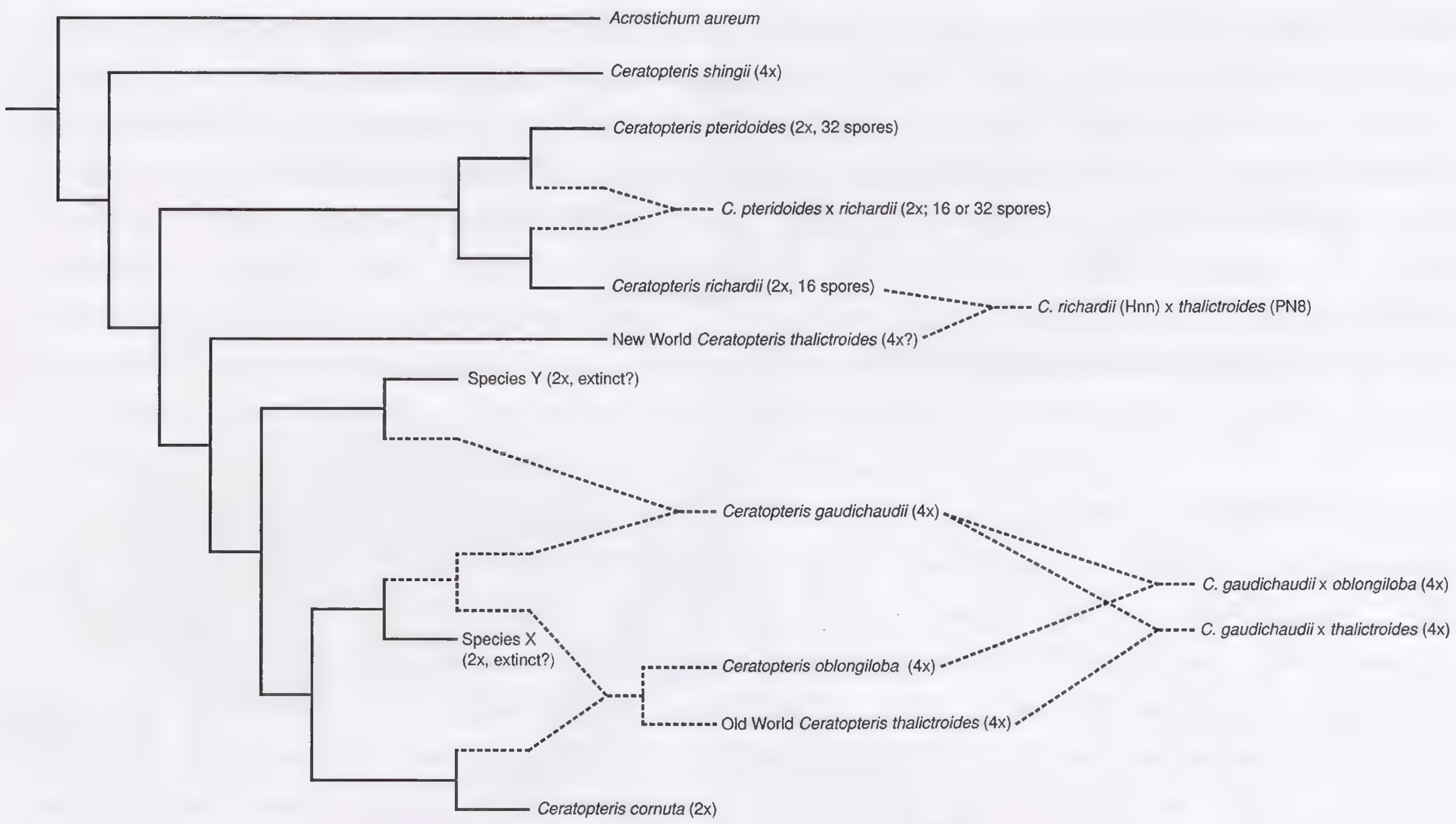
Acrostichum aureum
Ceratopteris shingii (4x)
Ceratopteris pteridoides (2x, 32 spores)
C. pteridoides x richardii (2x; 16 or 32 spores)
Ceratopteris richardii (2x, 16 spores)
C. richardii (Hnn) x thalictroides (PN8)
New World Ceratopteris thalictroides (4x?)
Species Y (2x, extinct?)
Ceratopteris gaudichaudii (4x)
C. gaudichaudii x oblongiloba (4x)
C. gaudichaudii x thalictroides (4x)
Species X
(2x, extinct?)
Ceratopteris oblongiloba (4x)
Old World Ceratopteris thalictroides (4x)
Ceratopteris cornuta (2x)

FIG. 2. Reticulogram showing hypothesized relationships in *Ceratopteris.* Solid lines show evolution by traditional diversification, dotted lines indicate hybridization. This diagram was created using phylogenies published by Adjie *et al.* (2007), Zhang et al. (2020), and Kinosian, Pearse, and Wolf (2020), and work by Hickok and Klekowski (1974), and Lloyd (1974). Species X and Y represent unknown or extinct diploid progenitor species, hypothesized by Adjie *et al.* (2007). Not included is *C. froesii*, a Brazilian endemic species, for which no genetic data exist.

to detect evidence of recombination in a dataset, separating it from other processes such as recurrent mutation and incomplete lineage sorting.

Utilizing this novel split network analysis of *Ceratopteris*, in conjunction with other published phylogenies of the group (Adjie *et al.*, 2007; Kinosian, Pearse, and Wolf., 2020; Zhang *et al.*, 2020), we drew a hypothesized reticulate network (Fig. 2). Our rationale for each branch or clade is outlined below:

- *Acrostichum* is the sister genus to *Ceratopteris* (PPG I, 2016), so it was used as the outgroup for our reticulogram.
- Zhang *et al.* (2020) showed that *Ceratopteris shingii* Y. H. Yan & R. Zhang is the sister to the rest of the genus, so we placed it as such in our reticulogram. This placement is discussed in detail below.
- Adjie *et al.* (2007) and Zhang *et al.* (2020) recovered *C. pteridoides* (Hook.) Hieron. and *C. richardii* as sister species; Hickok and Klekowski (1974) demonstrated the natural hybridization of these two lineages. We placed these two species as sister to one another on the next diverging branch, and indicated hybridization between them with dotted lines.
- We inferred the position of New World *C. thalictroides* from Kinosian, Pearse, and Wolf (2020), placing it sister to the Old World species in the genus. We also indicated hybridization between *C. richardii* and New World *C. thalictroides* as suggested by Nakazato (2007), which we discuss in more detail below.

- Work by Adjie *et al.* (2007) informed the hybrid relationships of *C. cornuta* (Pal. Beauv.) Le Prieur, *C. gaudichaudii* Brong., *C. oblongiloba* Masuyama & Watano, and Old World *C. thalictroides* (L.) Brong. Species Y, a putative progenitor of *C. gaudichaudii* suggested by Adjie *et al.* (2007), was placed as the first diverging lineage of Old World *C. thalictroides*, with Species X (suggested by Adjie *et al.* [2007]) and *C. cornuta* forming a clade.
- There is evidence of hybrid backcrossing between *C. gaudichaudii* and *C. thalictroides* (Masuyama, 2005; Kinosian, Pearse, and Wolf, 2020), as well as between *C. gaudichaudii* and *C. oblongiloba* (Masuyama, 2005). These reticulate events were added to the Old World *Ceratopteris* clade, detailed in the previous bullet point.
- The Brazilian endemic species *C. froesii* Brade may be closely related to New World *C. thalictroides* as they are both cryptic species of *C. thalictroides sensu lato*; however, no genetic information for this species is available, so it is not included in our reticulate network.

RESULTS

Raw data consisted of 2.58×10^6 raw reads per sample, with slightly more reads from silica-dried tissue compared to herbarium samples (See Online Supplementary Data 1). The final dataset of 51 individuals had 24% missing data. A Phi test found significant evidence for recombination in our dataset ($p = 6.513 \times 10^{-11}$). Our split network analysis was performed with a dataset of 26,593 SNPs, and separated each clade (species) of *Ceratopteris* into its own genomic group, but also revealed historical gene flow between all groups (Fig. 1). Using the same set of SNPs, we calculated Patterson's D for all combinations of three species plus the outgroup, and found evidence of gene flow within four groupings of species (Table 2). These results are congruent with previous studies that explored species relationships and hybridization within the genus (Adjie *et al.*, 2007; Kinosian, Pearse, and Wolf, 2020), but offer new insights on the prevalence of interspecific gene flow.

Our split network retrieves the same two clades of *Ceratopteris thalictroides* as Kinosian, Pearse, and Wolf (2020). Old World *C. thalictroides* is the most distinct species, as it is placed on a long branch in the split network, and has limited gene flow with other species as estimated by Patterson's D. In comparison, New World *C. thalictroides* also appears to be relatively distinct, but with potential shared ancestry with several other species. New World *C. thalictroides* was included in two of the four species groups with a significant Patterson's D, and potentially has shared ancestry with *C. cornuta*, *C. pteridoides*, Old World *C. thalictroides*, and *C. gaudichaudii* (Table 2).

The *Ceratopteris cornuta* 1 clade is relatively close to both Old World *C. thalictroides* and *C. gaudichaudii* in the split network; it also appears to have significant ancestral gene flow with other species (Table 2). Interestingly, the three individuals within the *C. cornuta* 1 clade seem to have slightly different evolutionary histories; these samples were from Tanzania, Oman, and Nepal,

TABLE 2. Estimation of gene flow between species of *Ceratopteris*. Four groups have significant gene flow, emphasized in bold. The program *Dsuite* (Malinksy, Matschiner and Svardal, 2020) was used to calculate the D-statistic from all possible quartets of species, including three ingroup taxa (P1-3, shown in this table) and one outgroup taxon (*Acrostichum aureum* for all groups, not shown) in each quartet. All species included in this study have gene flow with at least one other species as indicated by the D-statistics. This points towards rampant ancestral and/or modern gene flow throughout the genus. Specifically, *C. cornuta* has been hypothesized as a progenitor of *C. gaudichaudii* and Old World *C. thalictroides* (Adjie *et al.*, 2007), which is substantiated by these estimates of gene flow. We suspect that with a larger sample size, and more even sampling across species, we would detect additional evidence of gene flow between taxa.

P1	P2	P3	D-statistic	p-value
C. gaudichaudii	***C. cornuta***	***C. pteridoides***	**0.833002**	**0.00043111**
Old World *C. thalictroides*	*C. cornuta*	*C. gaudichaudii*	0.420208	0.0773966
New World *C. thalictroides*	*C. cornuta*	*C. gaudichaudii*	0.17197	0.292506
Old World *C. thalictroides*	***C. cornuta***	***C. pteridoides***	**0.868512**	**0.014955**
New World *C. thalictroides*	*C. cornuta*	*C. pteridoides*	0.291677	0.241956
Old World *C. thalictroides*	***C. cornuta***	**New World *C. thalictroides***	**0.737024**	**0.0326966**
Old World *C. thalictroides*	*C. gaudichaudii*	*C. pteridoides*	0.169435	0.346293
C. gaudichaudii	**New World *C. thalictroides***	***C. pteridoides***	**0.422691**	**0.0389534**
New World *C. thalictroides*	*C. gaudichaudii*	Old World *C. thalictroides*	0.145423	0.245784
Old World *C. thalictroides*	New World *C. thalictroides*	*C. pteridoides*	0.401949	0.136029

respectively. Since they are separated by such vast distances, slight genetic divergence is expected. There was also a second clade of *C. cornuta*, containing one individual of *C. cornuta*, and one unidentified specimen. Labeled as *C. cornuta* 2, it is very close to New World *C. thalictroides* and *C. pteridoides* in our split network. Both samples of the *C. cornuta* 2 clade are from within the known range of *C. richardii*, and may have been mis-identified by their collectors, or indicate undescribed variation with *C. cornuta*.

The outgroup, *Acrostichum aureum*, is most closely grouped with *C. pteridoides*. We chose to remove the outgroup from the final split network figure because it made the figure very difficult to read. *Acrostichum* was placed on an extremely long branch (~10x longer than any other branch), and the samples of *Ceratopteris* were clumped so close together it was impossible to see the relationships between them.

Perhaps the most interesting finding is that there is a significant amount of ancestral, but not recent, gene flow among all species, as indicated by the large network in the center of Fig. 1. This is also supported by Patterson's D, which found evidence of gene flow between four groups of species (Table 2), which are as follows: 1) *Ceratopteris gaudichaudii*, *C. cornuta*, and *C. pteridoides*; 2) Old World *C. thalictroides*, *C. cornuta*, and *C. pteridoides*; 3) New World *C. thalictroides*, *C. cornuta*, and Old World *C. thalictroides*; and 4) *C. gaudichaudii*, New World *C. thalictroides*, and *C. pteridoides*. All species included in this study have potential gene flow with at least one other species. Old World *C. thalictroides* and *C. gaudichaudii* were present in two of these groups; New World *C. thalictroides* and *C. cornuta* appeared three times.

We have combined the findings from our split network analysis, as well as work by Hickok and Klekowski (1974), Adjie *et al.* (2007), Masuyama and Watano (2010), Zhang *et al.* (2020), and Kinosian, Pearse, and Wolf (2020) to create an updated hypothesis of the reticulate relationships within *Ceratopteris* (Fig. 2).

DISCUSSION

Here we provide evidence for reticulate evolution within the model fern genus *Ceratopteris*. We build on previous work exploring hybridization and species concepts in the group (Adjie *et al.*, 2007; Lloyd, 1974; Masuyama and Watano, 2010), and provide the first hypothesis for the reticulate evolutionary history of the genus (Fig. 2). We find support for *Ceratopteris gaudichaudii* as a morphologically cryptic but genomically distinct species; separate New and Old World populations of *C. thalictroides*; plus eastern and western clades of *C. cornuta*, with the western clade (*C. cornuta* 2) potentially containing mis-identified specimens of *C. richardii*. We also find evidence of ancestral gene flow between species, as well as confirmation of more recent introgression (Fig. 1, Table 2; reviewed in Masuyama and Watano, 2005; Nakazato *et al.*, 2007).

Hybridization in context for a model system.—The neotropics are home to model species *Ceratopteris richardii*, as well as *C. pteridoides* and *C. thalictroides*. These species have received less attention than paleotropical species in recent molecular work, but there is some evidence of natural hybridization among New World taxa. Hickok and Klekowski (1974) showed that *C. richardii* hybridizes readily with diploid *C. pteridoides* in laboratory conditions, with approximately 60% spore viability. Natural hybrids between these two species have also been documented, with an intermediate morphology that sometimes looks similar to *C. thalictroides* (Hickok and Klekowski, 1974). Our split network results hint at the close relationship between these two taxa: the *C. cornuta* 2 and *C. pteridoides* clades are placed close to one another and have recent, but not on-going gene flow (Fig. 1). Three of four species groups found to have a non-bifurcating evolutionary history included *C. cornuta* and *C. pteridoides* (Table 2). However, we included *C. cornuta* as just one group when estimating gene flow: when the groups were separated, the sample size for each was too small to calculate Patterson's D. Therefore, we cannot comment on which group (1 or 2) is involved in this gene flow. Our results do, however, suggest that *C. richardii* and *C. pteridoides* might indeed be distinct species, yet lack reproductive boundaries to prevent interbreeding in natural conditions.

There is no evidence of recent admixture between *Ceratopteris richardii*, *C. pteridoides*, and New World *C. thalictroides* in our split network analysis, or the population structure analysis by Kinosian, Pearse, and Wolf (2020). We did, however, find evidence of gene flow between these species via Patterson's D, which can detect ancient as well as recent admixture (Durand *et al.*, 2011). This is supported by the fact that a few individuals of New World *C. thalictroides* (Th02, Th03, Th18, and Th25) are placed much closer to *C. pteridoides* than to other individuals of New World *C. thalictroides* (Fig. 1). Perhaps New World *C. thalictroides* is the result of a past hybridization event between *C. richardii* (or *C. cornuta*) and *C. pteridoides*, where polyploidization of the hybrid lineage has led to limited gene flow with its progenitors (see Otto and Whitton, 2000). Further investigation of this lineage will be important to understand the origins of New World *Ceratopteris*, and which species are actively hybridizing.

There is evidence that the Hnn lab strain of *C. richardii* can hybridize with the lab strain PN8, but with some postzygotic barriers (Nakazato *et al.*, 2007). Several authors have referred to PN8 as a collection of *C. richardii* with 32 spores per sporangium (Hickok, Warne, and Fribourg, 1995; Nakazato *et al.*, 2007); however, Lloyd (1974) described *C. richardii* as having 16 spores per sporangium, and *C. thalictroides* as having 32 spores per sporangium (Table 2). The original specimen of PN8 (*Nichols 1719*, GH; legacy.tropicos.org/Specimen/1771652) is labeled as *C. thalictroides*. The fact that there are postzygotic reproductive barriers between this specimen and *C. richardii* supports this original identification by Nichols, suggesting that PN8 is not *C. richardii*. However, inclusion of this sample and Hnn in future molecular work is critical for identification. The discrepancy in identification of PN8 emphasizes the importance of updating the *Ceratopteris* taxonomy. While lab organisms can be indispensable for many studies, it is important to be aware of their phylogenetic placement, and to understand the complexities that exist in defining species boundaries. This is exemplified by work on the phylogeny and biogeography of *Arabidopsis* after the publication of its reference genome (Beck, Schmuths, and Schall, 2008; Beck *et al.*, 2007).

Polyploidy and reticulation in Old World Ceratopteris.—Lloyd (1974) described *Ceratopteris thalictroides* as "highly polymorphic," and this seems to be an accurate description in both morphology and genomic composition (Kinosian, Pearse, and Wolf, 2020; Masuyama and Watano, 2010). Three cryptic species have been described from paleotropical plants (Masuyama and Watano, 2010), and there may be another cryptic species in the New World (Kinosian, Pearse, and Wolf, 2020), in addition to *C. froesii*.

The diploid species *Ceratopteris cornuta* has been hypothesized as an important progenitor of cryptic tetraploid taxa of Old World *Ceratopteris* (Adjie *et al.* 2007), but there is conflicting evidence concerning the evolutionary history of these taxa (Kinosian, Pearse, and Wolf, 2020). Adjie *et al.* (2007) hypothesized *C. cornuta* to be the paternal progenitor, and an unknown, potentially extinct, diploid referred to as Species X, to be the material progenitor of Old World *C. thalictroides*. However, the population structure analysis in Kinosian, Pearse, and Wolf (2020) does not indicate that either population of *C. thalictroides* (Old World or New World) has recently hybridized with, or is the result of recent hybridization between *C. cornuta* with any other species. In fact, the opposite is potentially true: *Ceratopteris cornuta* is the only species that has a large amount of admixture within its population structure (Kinosian, Pearse, and Wolf, 2020). In addition, our split network shows that Old World *C. thalictroides* is closely related to, but has not had recent gene flow with *C. cornuta* 1; this is evident by the long branch separating Old World *C. thalictroides* from the other species in the genus (Fig. 1). We did, however, find support that Old World *C. thalictroides* and *C. cornuta* may share a historic, reticulating history (Table 2; see Durand *et al.* 2011). In addition, it is possible that progenitor Species X is extinct, or perhaps that we have just not sampled it. Our data processing software (*ipyrad*) selects loci based on similarity throughout the dataset (Eaton and Overcast, 2020). If the progenitor(s) of Old World *C. thalictroides* were not included in this study, the loci only present from its progenitors and not shared with other species may have been omitted.

A second tetraploid species thought to have arisen from a hybridization event between *C. cornuta* and Species X is *Ceratopteris oblongiloba* (Adjie *et al.*, 2007; Fig. 2). However, in this case *C. cornuta* is the maternal progenitor, and the unknown diploid Species X is the paternal progenitor of *C. oblongiloba*. These two species may be the result of reciprocal crossing between two diploids forming two distinct polyploid species. Another instance of reciprocal crossing between two diploids is known from the allotetraploid species *Polypodium hesperium* (Haufler *et al.*, 1995). There are two allopatric populations of *P. hesperium*, identified by their plastid haplotypes; this may be a result of different genomes conferring advantages in separate habitats (Sigel, Windham, and Pryer, 2014). In the case of *C. oblongiloba* and Old World *C. thalictroides*, perhaps these reciprocal polyploids have had time to diversify both morphologically and ecologically since their hybrid origin.

The hybrid origin of *Ceratopteris gaudichaudii* does not seem to involve *C. cornuta* (Adjie *et al.*, 2007), although they may have shared ancestry (Fig. 1; Table 2). Adjie *et al.* (2007) hypothesized this allotetraploid was derived from two potentially extinct diploids: Species X and Y (Fig. 2). In addition, backcrossing has been described between *C. gaudichaudii* and *C. oblongiloba* (Masuyama and Watano, 2005); and between *C. gaudichaudii* and Old World *C. thalictroides* (Kinosian, Pearse, and Wolf, 2020; Masuyama and Watano, 2005; Fig. 2). Perhaps because *C. gaudichaudii* shares one progenitor (Species X) with *C. oblongiloba* and Old World *C. thalictroides*, their genomic similarity enables these hybrid species to reproduce with one another, although chromosome pairing is not always complete (Masuyama and Watano, 2005).

The oldest fossil from the Ceratopteridoideae sub-family is estimated to be about 42-56 My old (Bonde and Kumaran, 2002; Dettmann and Clifford, 1992; Rozefelds *et al.*, 2016), so there is potential for some deep divergences, perhaps with more recent hybridization creating the complex relationships we see today. A similar pattern of hybridization across deep time scales is seen in North American *Dryopteris* (Sessa, Zimmer, and Givnish, 2012a; 2012b), which is also known to have an extinct diploid progenitor species, important in the hybrid origin of three taxa (Sessa, Zimmer, and Givnish, 2012b). Further investigation into divergence times, and progenitor Species X and Y of Old World *Ceratopteris* is important to understand the timeline of reticulate evolution in the genus.

Outlying taxa.—The recently discovered *Ceratopteris shingii* was not included in this study, but Zhang *et al.* (2020) did not find any evidence of hybridization in this lineage. *Ceratopteris shingii* is endemic to the island province of Hainan in southern China; molecular evidence shows that it is sister to all other species in the genus. The study by Zhang *et al.* (2020) used both Bayesian inference (MrBayes) and maximum likelihood (RAxML) to reconstruct a plastid phylogeny for the genus. Both trees showed high support for *C. shingii* as sister to the rest of the genus; however, the relationships between the remaining taxa had less support. Bayesian inference and maximum likelihood methods are often in accordance, but they can disagree in some cases, such as very short branch lengths (Alfaro, Zoller, and Lutzoni, 2003). A potential explanation is that *C. shingii* represents a distinct morphological and genetic lineage, exemplified by its unique creeping rhizomes (Zhang *et al.*, 2020). The remaining species in *Ceratopteris* may be the result of a recent, rapid radiation, which would explain the discordance between the Bayesian inference and maximum likelihood trees.

The Brazilian endemic *Ceratopteris froesii* is the only species for which we were neither able to infer its phylogenetic position from other studies, nor find any available material. Masuyama and Watano (2010) described *C. froesii* as a cryptic species of *C. thalictroides*, characterized by very small plants (~3cm tall) known only from a few localities in Brazil. Including this unusual species in future research would help to better understand New World diversity of

Ceratopteris, especially in the context of morphological plasticity. Masuyama (1992) found that different populations of Japanese *Ceratopteris* had distinct morphologies in the wild, but the phenotype of their progeny could be altered by manipulating greenhouse conditions. There is some evidence that *C. froesii* maintains its small size in cultivation, but it would be interesting to repeat the work done by Masuyama (1992) on South American *Ceratopteris*, to see if the New and Old World species have a similar level of morphological plasticity.

Conclusion

The present study builds on previous work on *Ceratopteris*, and investigates reticulate relationships that do not fit a traditional bifurcating tree. The split network we generated recovered similar relationships to previously studies, and shows the large amount of historical gene flow between all species of *Ceratopteris*: the web-like nature of the center of the network indicates that there was past hybridization, leading to conflicting evolutionary histories of different loci (Fig. 1). Estimation of Patterson's D also confirms ancestral gene flow between almost all species in the genus (Table 2). These connections help explain the complicated relationships between many species of *Ceratopteris*, and why some species hybridize so readily today (*e.g.*, Hickok and Klekowski, 1974). For future work, it will be important to include both strains of the model species *C. richardii*, as well as the newly described *C. shingii*. The taxonomic and evolutionary relevance of these species will hopefully help us better understand the complex history of this genus.

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Appendix A.

Online Supplementary Data 1: Specimen collection information

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Diversity Patterns of Neotropical Ferns: Revisiting Tryon's Centers of Richness and Endemism

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Abstract.—Almost fifty years ago Dr. Rolla M. Tryon investigated the patterns of neotropical fern diversity and discovered that sites of exceptional richness and endemism are found in five predominantly montane regions. Here, we revisit these sites with the aid of contemporary methodologies. We integrate phylogenetic, ecological, climatic, and occurrence data to better understand what factors contribute to the patterns of fern diversity throughout the neotropics. With this dataset we are able to reassess Tryon's neotropical hotspots fifty years later and take one step closer to understanding the processes governing the distribution of fern species. We recover six hotspots of neotropical species richness and endemism that closely mirror those delineated by Tryon. Like Tryon, we find that hotspots are found predominantly in montane regions with more climatic space compared to surrounding areas. Patterns of species richness and lineage diversification can largely be explained by the extent of available habitats, especially in association with montane ecosystems. We also show that patterns of species assemblages across the neotropics are largely dictated by distance and elevation. In synthesis, we propose that, in addition to migration and persistence of relictual lineages, patterns of species richness and endemism in the neotropics are driven by *in situ* speciation in montane regions.

Key Words.—biodiversity, species richness, endemism, macroevolution, diversification, neotropics, ferns

Explaining the uneven distribution of species around the world is a major challenge in biogeography (Humboldt and Bonpland, 1805; Perrigo *et al.*, 2020). Within the tropics, a disproportionate number of species and endemics are found in montane regions (Korner, 2000; Hoorn *et al.*, 2013; Antonelli *et al.*, 2018), yet mechanisms explaining these patterns remain poorly understood (Rahbek *et al.*, 2019a; b; Perrigo *et al.*, 2020). Leading investigations, however, indicate that topographic complexity, ecological variability, broad climatic space, and low seasonality are important factors in building biodiversity hotspots (Rahbek *et al.*, 2019a).

On a macroevolutionary scale, hotspots can originate through a combination of mechanisms including high speciation, low extinction, and immigration (Wiens and Donoghue, 2004; McKenna and Farrell, 2006; Jablonski, Roy, and Valentine, 2006; Harrison and Noss, 2017). Young mountain ranges, such as

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the Tropical Andes in South America and the Hengduan Mountains in East Asia are two such global hotspots that harbor rapidly radiating lineages (Givnish *et al.*, 2015; Lagomarsino *et al.*, 2016; Xing and Ree, 2017; Testo *et al.*, 2019; Ye *et al.*, 2019). Species diversification within these sites is attributed to recent cycles of geologic uplift over the last 30 million years, leading to the development of novel habitats such as the high elevation alpine zones (Gregory-Wodzicki, 2000; Garzione *et al.*, 2008). Steep mountains like these in tropical ecosystems have many different habitats stacked within a relatively small geographic space, and the seasonality within these habitats is low (Janzen, 1967). Species within these ecosystems tend to have small range sizes (McCain, 2009) because reduced seasonal climatic variation leads to smaller physiological tolerances among these species, limiting successful dispersal along elevational gradients. On a microevolutionary scale, reduced gene flow between different populations in these stacked habitats may increase reproductive isolation, and eventually lead to speciation (Polato *et al.*, 2018). For this reason, tropical mountains are expected to harbor more rapidly diversifying lineages. To understand these processes on a macroevolutionary scale, integrative studies incorporating phylogenetic, climatic, topographic, and georeferenced occurrence data are needed, but for many groups, like ferns, these broad datasets have remained sparse until relatively recently (Suissa, Sundue, and Testo, in review; Kessler *et al.*, 2011; Testo and Sundue, 2016; Lehtonen *et al.*, 2017; Weigand *et al.*, 2019).

For at least 100 years, researchers have identified specific regions of tropical America (the neotropics) as predominant centers of fern diversity (Christ, 1910). In 1972, Dr. Rolla M. Tryon set out to study the broad patterns of neotropical fern diversity within these areas. In the absence of quantitative tools available today, Tryon turned to monographs and herbarium records. From these, he calculated species richness and endemism within the neotropics and concluded that there are five fern biodiversity hotspots: Mexico, Mesoamerica, the Tropical Andes, the Guiana Highlands, and Southeastern Brazil. Of these five hotspots, Tryon determined that three of them (Tropical Mexico, the Tropical Andes, and Southeastern Brazil) are the richest in species and endemics. Tryon also documented the number of overlapping species between regional biodiversity hotspots and found that regions closer together have similar community assemblages. From these results he speculated that, in addition to migration and vicariance, neotropical fern diversity is largely shaped by speciation through ‘peripheral divergences’ or peripatric speciation (Grant, 1971; Tryon, 1972, 1985). Since Tryon’s work 50 years ago, advancements have been made in our understanding of regional (Moran, 1995; Kluge, Kessler, and Dunn, 2006; Watkins *et al.*, 2006; Prado *et al.*, 2015; Salazar *et al.*, 2015; Khine *et al.*, 2019), as well as global (Moran, 2008; Kessler *et al.*, 2011; Weigand *et al.*, 2019) patterns of fern biodiversity. Here, we add to this work with a specific re-investigation of the patterns and drivers of neotropical fern diversity.

We use a recent time-calibrated phylogeny and georeferenced occurrence data to identify hotspots of fern richness, endemism, and diversification within the neotropics. We incorporate climatic, topographic, and edaphic data to contextualize these patterns, leading to a better understanding of the underlying factors driving neotropical fern diversity. Following Tryon, we ask: Where are the neotropical fern biodiversity hotspots? Why might these sites be disproportionately species rich? What might be ecologically distinct about these sites compared with surrounding regions? And, what are the patterns of species community assemblages in distance and elevation across the neotropics?

Materials and Methods

Occurrence data.—Species occurrences were downloaded from the Global Biodiversity Information Facility (GBIF, www.gbif.org). We restricted the search to georeferenced herbarium specimens within the neotropics. Available occurrence records contain numerous taxonomic and nomenclatural issues. In order to generate a useful dataset, we thoroughly cleaned the data to minimize these problems. First, we generated a consistent taxonomic framework across our datasets by synonymizing names based upon a recent taxonomic fern database (Hassler and Schmitt, 2018). Most cases of synonymy were easily handled, however, because homonyms caused issues, we paid close attention to these in order to avoid incorrect assignment of names to occurrence records. Second, data points were rigorously cleaned in multiple steps. First, we flagged and removed problematic records using the CoordinateCleaner package (Zizka *et al.*, 2019) in R v. 4.0 (R Core Team, 2018). This step removes erroneous and overly simplified coordinates such as those mapped to the centroid of a country or state. Then, we generated interactive maps for each species using the rbokeh package (Hafen and Continuum Analytics, 2016). Each species map was manually inspected and assessed based on our knowledge of each species' distribution. This step allowed us to flag any outlier occurrence for closer inspection. Problematic occurrence points were removed from the dataset. In total we removed around half of the original occurrence records leaving us with a final, cleaned dataset consisting of 288,944 occurrence records corresponding to 3,358 species.

Spatial analyses.—We associated our cleaned occurrence records with 1°×1° ($\sim$111 km^2) grid cells using QGIS 3.10.1 and trimmed the resulting data to retain a single occurrence of each species present per grid cell. We used degree projections instead of an equal-area projections because these two metrics converge at tropical latitudes. For each grid cell, we first calculated species richness and weighted endemism (Williams and Humphries, 1994), using custom scripts in R. We then extracted lineage-specific speciation rates for each taxon from a recent time-calibrated phylogeny (Suissa, Sundue, and Testo, in review; Testo and Sundue, 2016). We used these to calculate mean speciation rates of lineage assemblages per grid cell; this metric is the mean of the lineage specific speciation rate for each individual species occurring within a single grid cell. We then used Biodiverse v. 3.00 (Laffan, Lubarsky, and Rosauer, 2010) to calculate phylogenetic diversity (PD) and phylogenetic endemism (PE). Phylogenetic diversity is the sum of the total branch lengths of the phylogeny connecting species within a given area (Faith, 1992). Phylogenetic endemism is the range size corrected equivalent of PD (Rosauer *et al.*, 2009).

Grid cells of exceptional species richness and endemism were statistically identified using the Gi* local statistic (Getis and Ord, 1996) in QGIS with the Hotspot Analysis plugin (Oxoli, Prestifilippo, and Bertocchi, 2017). For a more detailed discussion of these methods see Suissa, Sundue, and Testo (in review). In short, this program quantifies and computes the Z-score and p-values of the Gi* local statistic, assuming a complete random spatial hypothesis. The output indicates whether a 1°×1° grid cell is positive and significant (high richness and endemism relative to a null model), or not. Resulting hotspots comprised all of the positive and significant 1°×1° grid cells within each region. In the case of Tropical Mexico, Mesoamerica, and the Tropical Andes, all resulting 1°×1° grid cells within these sites were contiguous. We demarcated Tropical Mexico and Mesoamerica by geological boarders (Isthmus of Tehuantepec). Similarly, we chose to separate Mesoamerica and the Tropical Andes at the Darién Gap.

Ecological variables.—Using data from CHELSA (Karger *et al.*, 2017), SoilGrids (Hengl *et al.*, 2017), and USGS GMTED2010 (Danielson and Gesch, 2011), we generated climate, soil, and topographic data, respectively, for each grid cell. Within each grid cell we generated ecological variables directly from individual species occurrence coordinates. This was done to prevent generating erroneous metrics for sites that harbor no fern species, *e.g.*, extremely high altitudes. The specific ecological variables used in our downstream analyses included: mean annual temperature, minimum temperature of the coldest month, annual precipitation, precipitation seasonality, soil pH, soil organic carbon content, and mean elevation. All data layers were associated with the 1°×1° grid cells and data values were extracted using QGIS. Environmental niche heterogeneity was calculated as the mean of the standard deviations of each environmental variable within each grid cell. To investigate the relationship between richness, speciation rates and environmental niche heterogeneity we conducted an ordinary least squares (OLS) regression and to account for spatial autocorrelation we used a simultaneous autoregressive (SAR) lag model. We used the number of nearest neighbors in our weight matrix. We then compared the SAR vs OLS model with Akaike information criterion (AIC). Comparisons can be found in the supplemental dataset.

Comparison of regional data.—To quantify similarity between regional hotspots we amalgamated all the data for each significant 1°×1° grid cell within each hotspot. We then calculated total species richness, endemism, percent of total neotropical species richness found within each region, and percent regional endemism. We directly compared data from Tryon (1972) to ours using bar graphs. To assess the distribution of species across elevational gradients, we first binned species occurrences into seven elevational groupings by meters (0–500 m, 501–1000 m, 1001–1500 m, 1501–2000 m, 2001–2500 m, 2501–3000 m, 3000 m+). Then using QGIS we estimated the area of each elevational zone in the neotropics. We also calculated the species richness per area (10,000 km^2) within each elevational zone. We plotted species richness, endemism, phylogenetic diversity, phylogenetic endemism, and mean speciation rates of lineage assemblages per grid cell in the neotropics using QGIS. In order to draw broader conclusions about neotropical fern biodiversity we compared richness, endemism, speciation rates of lineage assemblages, and environmental heterogeneity within each 1°×1° grid cell. Statistical comparisons between grid cells in each region were made using an analysis of variance. Boxplots were overlaid around the raw points, the middle line in the boxes represent the median, the lower and upper edges of the box correspond to the first and third quartiles, respectively. The upper and lower whiskers extend to 1.5-times the inter quartile range. The data outside of the whiskers are considered outliers. The asterisks indicate significant differences based on a t test, ***p= 0.001, **p=0.01, *p=0.05, NS not significantly different.

Distribution patterns.—Species affinities were calculated in two ways. First, species lists were made for each broad hotspot and species lists within each hotspot were compared using a custom script in R. To further investigate the relationship between species affinities and distance in the neotropics, we generated species lists for each 1×1-degree grid cell and conducted an all-by-all comparison between species in each grid cell. We then calculated the geodesic distances between each of the compared grid cells using the distm function in the geosphere package in R (Hijmans *et al.*, 2017). In order to model the relationships between grid cell-level species affinities and distance we fit a polynomial model of order 1–3 and tested their goodness of fit (Supplementary dataset). The best fitting model was chosen based on optimizing the AIC by calculating the delta AIC using the akaike.weights function in the qpcR package (Ritz and Spiess, 2008). Finally, we investigated the three-dimensional relationship between distance, elevation, and shared similarity of species between each site. To do this we regressed the average grid cell elevation between compared sites on the y and z axis, and distance between sites on the x axis. We then colored each point by its percent of shared species. This was carried out using the plot_ly function in the Plotly package in R (Sievert, 2018).

RESULTS

We determined which 1°×1° grid cells were disproportionately high in species richness and endemism compared to a null model in order to identify neotropical hotspots (Fig. 1a). We identified a total of 349 grid cells distributed throughout six distinct hotspots: the Greater Antilles, Tropical Mexico, Mesoamerica, the Tropical Andes, the Guiana Highlands, and Southeastern Brazil. Most of these regions align with Tryon's circumscription (Fig. 1b). Species richness values for our sites (number of species; percent of total neotropical species richness) were greatest in the Tropical Andes (1967; 58%), followed by Mesoamerica (1362; 40%), Southeastern Brazil (890; 27%),

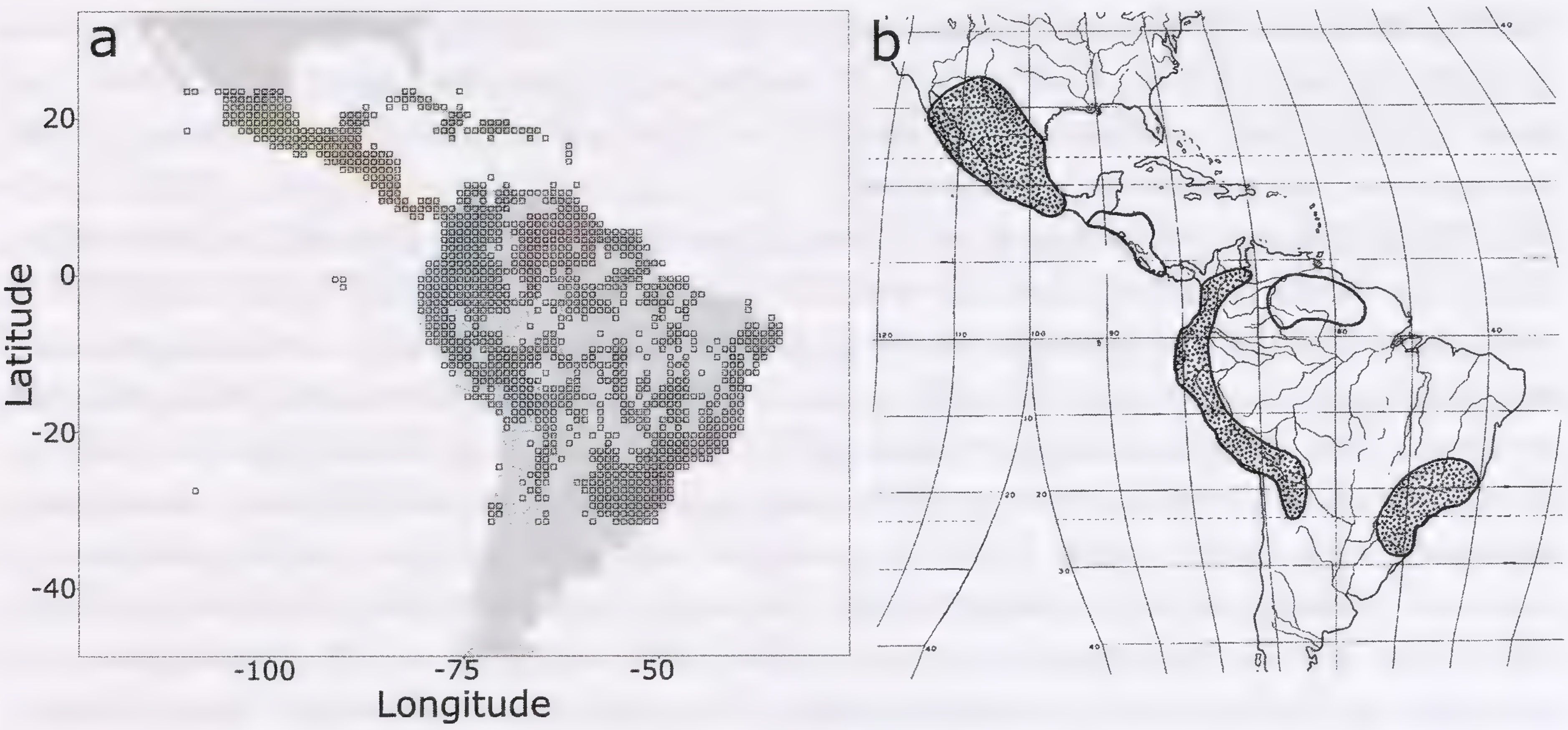

FIG. 1. a. Six hotspots circumscribed in this study. Colored squares correspond to 1°×1° grid cells identified as having high endemism, species richness, or both. Each grid cell is colored to correspond to their regional hotspot: Tropical Mexico (yellow); Mesoamerica (orange); Greater Antilles (green); Tropical Andes (blue); the Guiana Highlands (red); Southeastern Brazil (purple). b. Regional hotspots circumscribed by Tryon (1972). Stippled regions are 'primary' sites of endemism and richness, while non-stippled regions are 'secondary' sites. Figure 1b modified from Tryon,1972 with permission from John Wiley & Sons.

Tropical Mexico (840; 25%), the Greater Antilles (799; 24%), and the Guiana Highlands (757; 23%) (Fig. 2a). Percent of regional endemism (percent of unique species within each hotspot) was highest in the Tropical Andes (25%), followed by the Greater Antilles (23%), Southeastern Brazil (15%), Mesoamerica (14%), Tropical Mexico (8%), and the Guiana Highlands (4%) (Fig. 2b). On a per area basis we find that the Tropical Andes and Mesoamerica have the highest species richness while the Guiana Highlands have the lowest species richness within circumscribed hotspots (Fig. 3a, b). The number of endemic species per broad hotspot varied from 31 in the Guiana Highlands to 495 in the Tropical Andes (Supplementary dataset). Mean comparisons based on a one-way ANOVA suggested that there were no differences between average species richness in grid cells in the Tropical Andes, Greater Antilles, and Tropical Mexico (Fig. 3a). We also find that species richness on a per area basis peaks at 2500–3000 meters in the neotropics (Fig. 3b). Furthermore, grid cells in the Guiana Highlands and Southeastern Brazil do not differ significantly from each other and on average were lower than all other regional hotspots. The Tropical Andes, Greater Antilles, and Mesoamerica are the sites of highest endemism with the neotropics (Fig. 2b; Fig. S1), while the Guiana Highlands and Tropical Mexico were found to have the lowest endemism within hotspots.

The highest proportion of shared species between Mesoamerica and Tropical Mexico (30%), followed closely by Mesoamerica and the Tropical Andes (28%). Between regional hotspots, the percent of shared species tended to decrease with distance (Fig. 4a). We examined these patterns of species

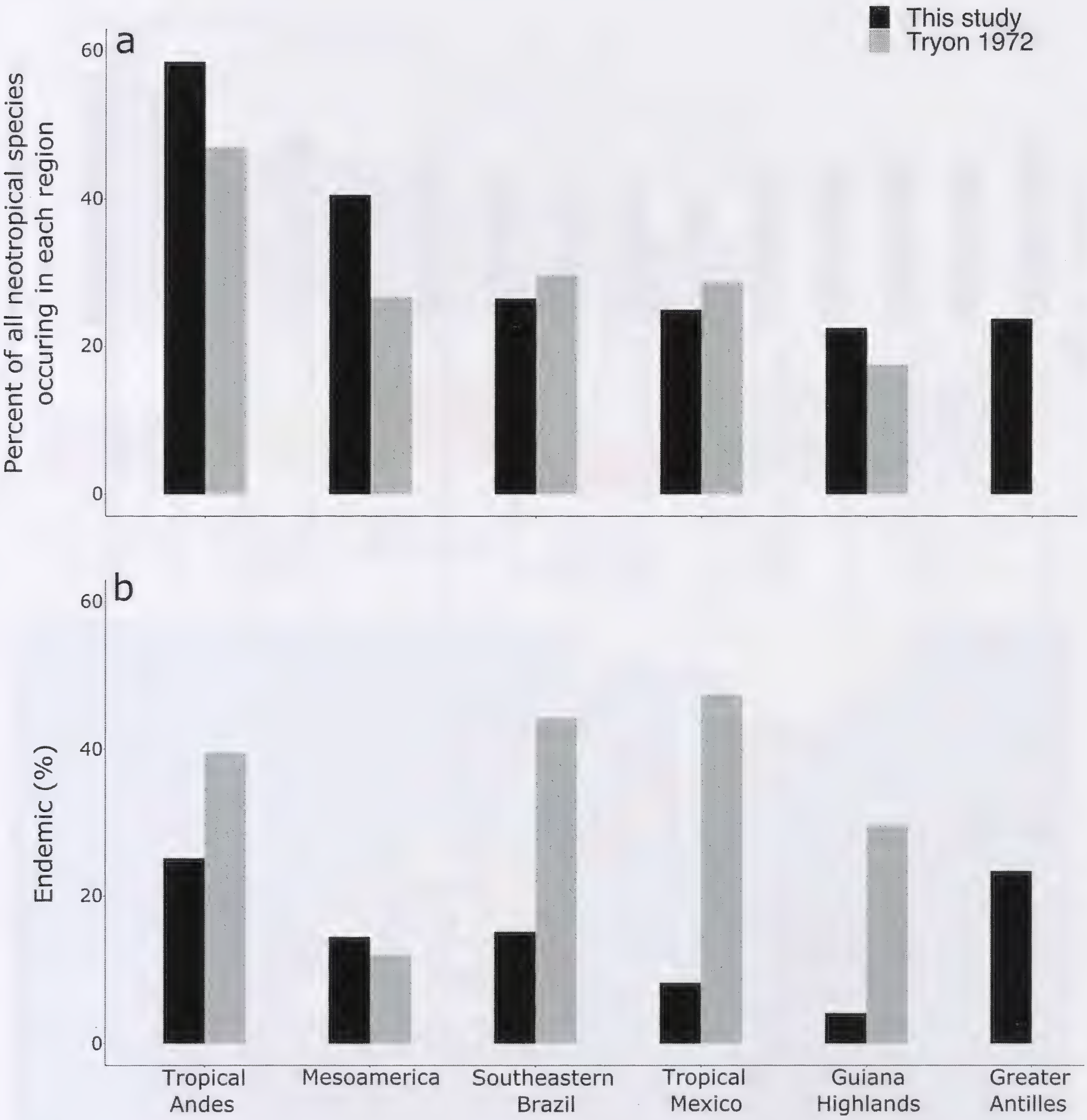

FIG. 2. a. Regional richness (as the percent of the all neotropical species found within a given hotspot) recovered in this study (black bars) and Tryon 1972 (gray bars). b. The percent of the total flora within each region that is endemic in this study (black bars) and Tryon 1972 (gray bars).

assemblages within 1°×1° grid cells across the entire neotropics. We found that, based on AIC the third-order polynomial model best fit the data, and has a correlation coefficient of 0.24, a p-value of 0.001, and a delta-AIC of 0 (Fig. 4b). The relationship between shared species and distance can be explained as an initial linear decrease in shared species as distance increases, followed by a long asymptotic trend and a final terminal decrease (Fig. 4b). In addition to distance, we find that elevational differences between grid cells is important in determining shared species overlap (Fig. S2). Specifically, sites that are closer together, but occur at dramatically different elevations have more distinct fern floras compared with those at similar elevations.

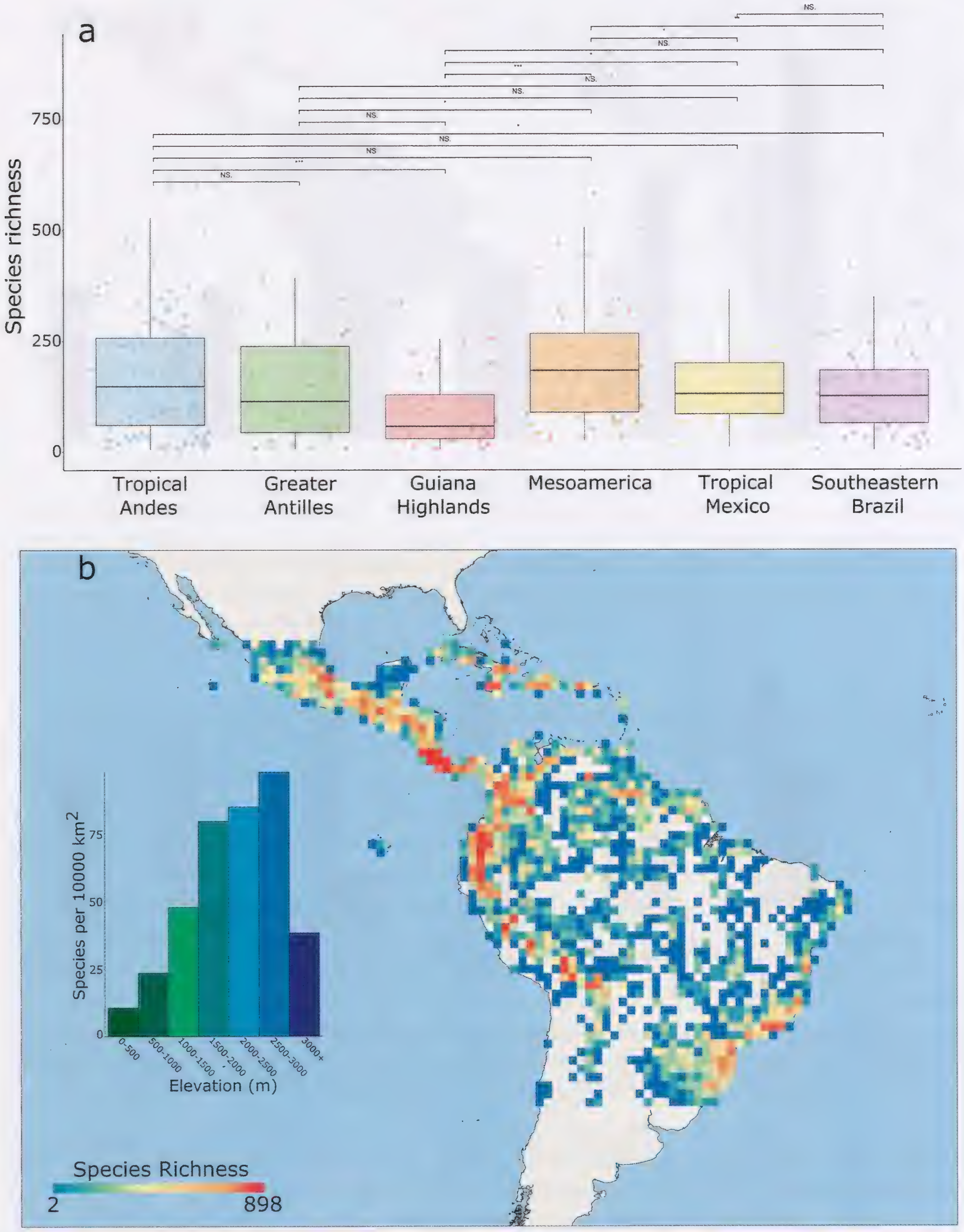

FIG. 3. a. Boxplots of species richness per 1°×1° grid cell in each hotspot. Points indicate values for individual grid cells. Boxes indicate interquartile range of values, darker line within box indicates mediate values, whiskers indicate 1.5× interquartile range. Brackets with '*', '**', or '***' indicate significant differences (p, < 0.05, $p < 0.01$, or $p < 0.001$), NS indicates that the means are not significantly different. b. Fern species richness estimated from 288,944 georeferenced herbarium records. Colored cells indicate the estimated species richness within 1°×1° grid cell. Inset bar graph indicates species richness per 10000km^2 at different elevational zones throughout the neotropics.

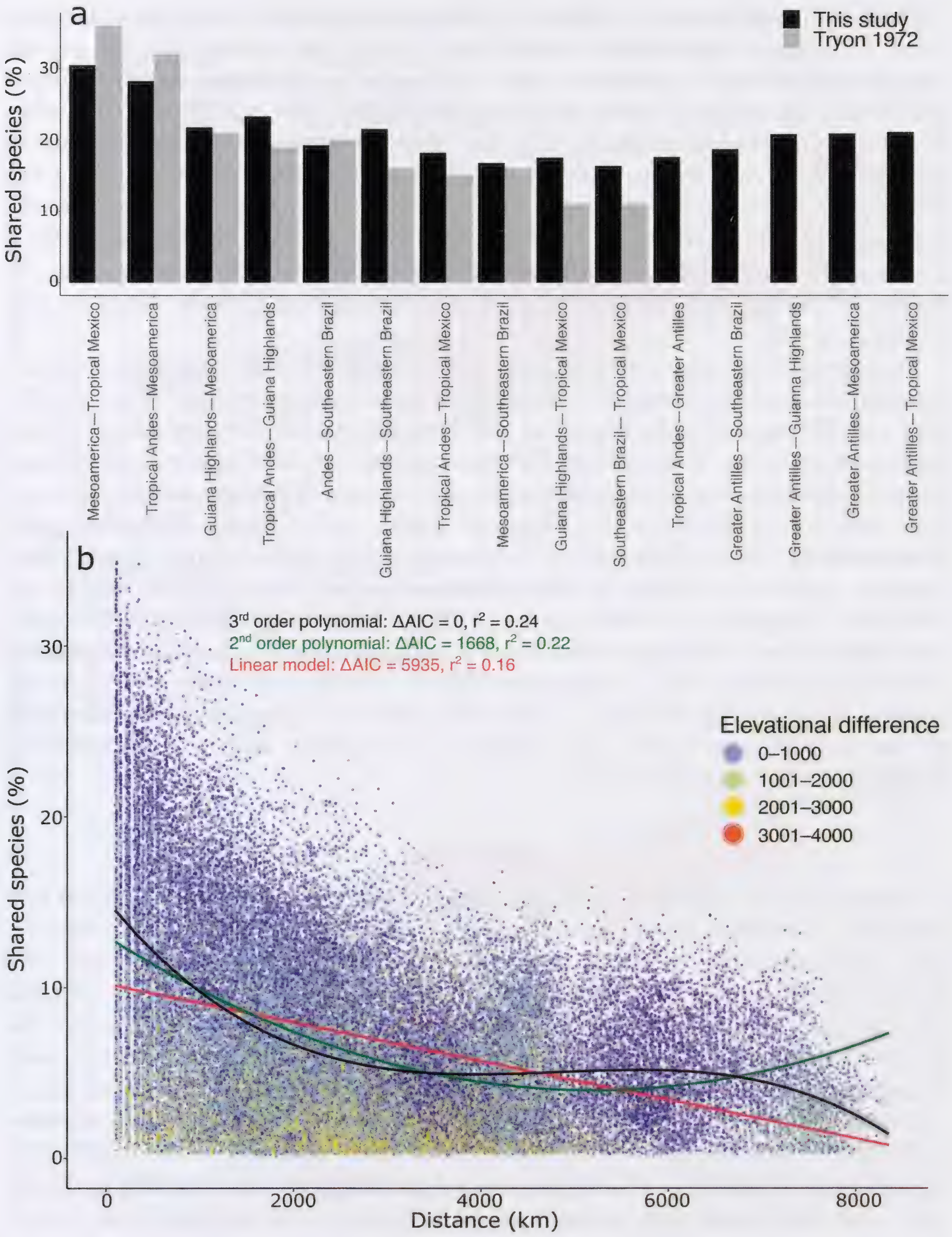

FIG. 4. a. The percent of shared species between each regional center in this study (black bars) and Tryon 1972 (gray bars). b. Each point represents the percent of shared species between two 1°×1° grid cells throughout all of the hotspot regions. The points are situated along the x-axis based on the geodesic distance between the two points. Each point is colored by the difference in the average elevation between each 1°×1° grid cell.

We then investigated the effects of niche heterogeneity on mean speciation rates of lineage assemblages within grid cells. We determined an overall correlation between speciation rates of lineage assemblages within hotspot grid cells and environmental niche heterogeneity ($p = 0.001$) in all hotspots excluding Southeastern Brazil (Fig. 5a). Furthermore, we found that species richness positively scales with niche availability within all hotspots in the neotropics ($p < 0.05$) (Fig. 5b). Environmental niche heterogeneity and topographic heterogeneity within 1°×1° grid cells were all significantly higher in hotspots compared with adjacent non-hotspot sites (Fig. 5a; Fig. S3a, b). However, results are sensative to area circumscription (Suissa, Sundue, and Testo, in press).

Phylogenetic diversity and phylogenetic endemism were higher in circumscribed biodiversity hotspots compared to surrounding regions (Fig. S4). Per grid cell PD tended to be higher in the Tropical Andes, Greater Antilles, and Mesoamerica (Fig. S1a), while PE was highest in the Greater Antilles and Tropical Mexico (Fig. S1c). Average speciation rates of lineage assemblages per grid cell was highest in the Tropical Andes, the Guiana Highlands, and Southeastern Brazil (Fig. S1b). Compared with surrounding non-hotspot regions, speciation rates of lineage assemblages were higher within all hotspots compared to surrounding regions (Fig. S3b). Between hotspots, speciation rates of lineage assemblages were highest in the Tropical Andes, the Guiana Highlands, and Southeastern Brazil, while they were lowest in the Greater Antilles (Fig. S4; Fig. S1). Overall, patterns of phylogenetic endemism, diversity, and speciation are found to be highest within circumscribed biodiversity hotspots (Fig. S4).

Discussion

Almost half a century after Tryon's original hypotheses on neotropical fern diversity, we revisit these questions with contemporary methods. We identify six predominantly montane sites of exceptional species richness and endemism (Fig. 1a). In decreasing order of species richness these include: the Tropical Andes, Mesoamerica, Southeastern Brazil, Tropical Mexico, the Greater Antilles, and the Guiana Highlands (Fig. 1a; Fig. 2a). Within each hotspot we find that species richness scales linearly with the availability of niche space (Fig. 5b). A combination of factors including the exceptional topographic relief in these sites (Fig. S3a), the mid- to upper- elevational peaks of species richness (Fig. 3), and the species-depauperate surrounding lowlands (Fig. 3b), indicate the importance of mountains in maintaining tropical fern diversity, as discussed by previous authors (Barrington, 1993; Kluge, Kessler, and Dunn, 2006; Weigand *et al.*, 2019). We show that these montane hotspots harbor a disproportionate amount of environmental niche space (Fig. 5a), and we propose that the main, but not exclusive, underlying mechanisms explaining the patterns of neotropical fern diversity is *in situ* species proliferation within tropical mountains. While some of these observations have been acknowledged qualitatively for over 100 years (Christ, 1910), our

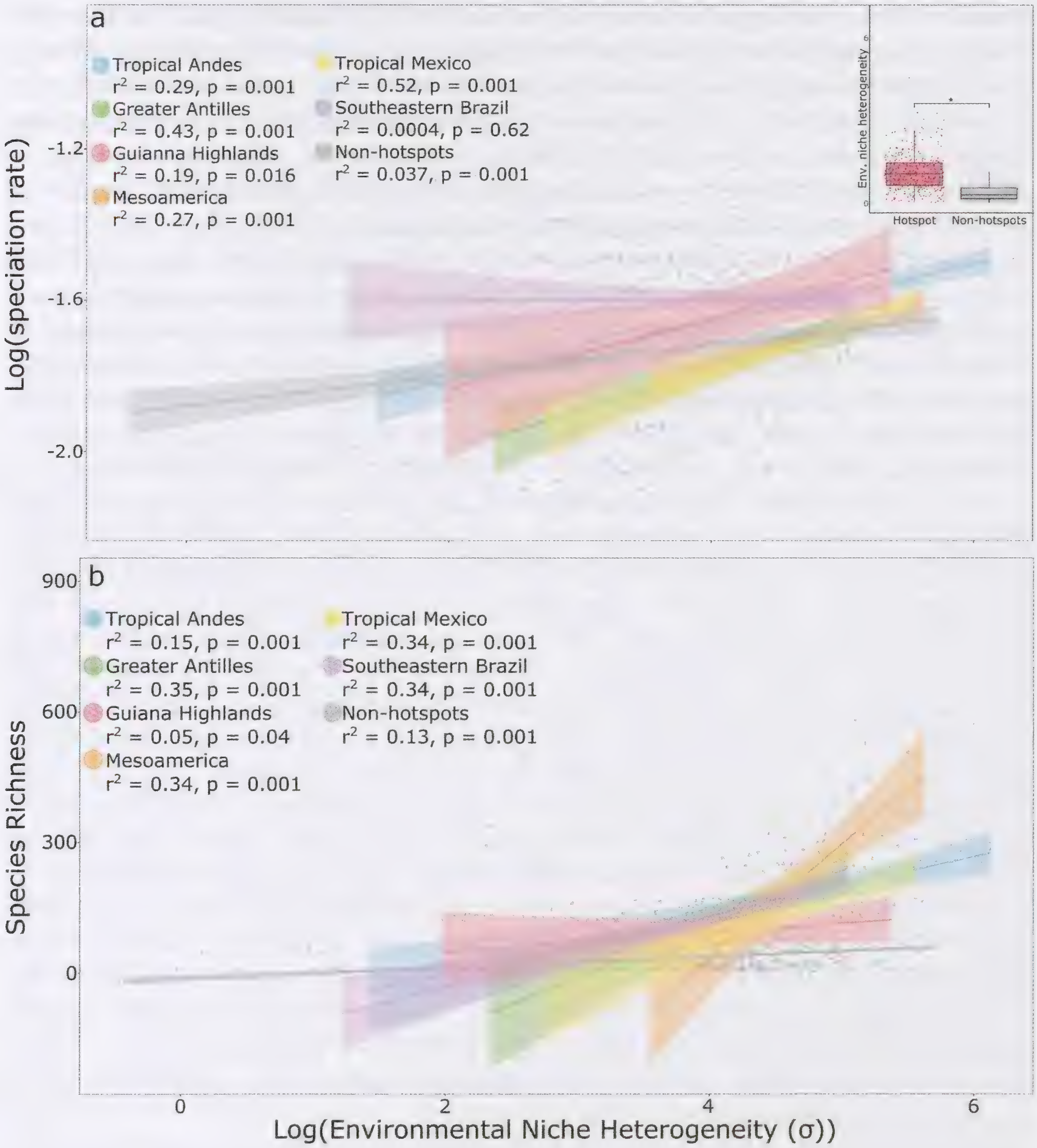

FIG. 5. a. Correlation between speciation rates of lineage assemblages and environmental niche heterogeneity within each grid cell in hotspots (colored) and non-hotspots (gray). Inset boxplot shows environmental niche heterogeneity estimates within hotspots (red) and non- hotspots (gray); asterisks indicate significant differences ($p < 0.05$). b. Correlation between species richness and environmental niche heterogeneity in hotspots (colored) and non-hotspots (gray). Points correspond to values from 1°×1° grid cells and colors correspond to individual hotspots; lines indicated best-first linear regressions with 95% confidence intervals.

data quantify these historical patterns, deepening our understanding of neotropical fern diversity.

Despite methodological differences, our results correspond closely with those generated by Tryon's tabulations from herbarium specimens and monographs (Fig. 1b). Sites identified in common include Tropical Mexico, Mesoamerica, the Tropical Andes, the Guiana Highlands, and Southeastern Brazil. The congruence of our results attests to the robustness of these sites as true hotspots of neotropical fern biodiversity. While the broad circumscription of hotspots is similar, there are nuanced differences between the results in our dataset compared with those of Tryon. For instance, the total number of species circumscribed in our hotspots are greater than what he found (Supplementary dataset), our patterns of endemism differ (Fig. 2b), and we identified the Greater Antilles as a hotspot which he did not (Fig. 1). While Tryon acknowledged the exceptional richness and endemism in the Greater Antilles, for reasons unknown to us, he did not circumscribe it as a hotspot in 1972. We attribute most of our differences to the biases in the two datasets, including the the number of taxa used and herbarium specimens available. Furthermore, Tryon was known to 'lump' rather than 'split' species (D. S. Barrington, pers. com.), which may have also led to some discrepancies.

Another prominent difference between our results is that Tryon identified interstitial regions occurring between hotspots as being putatively lower in species richness and endemism (Fig. 1b). We do not find such regions between our areas of Tropical Mexico, Mesoamerica, and the Tropical Andes; we find that the region spanning from the Tropical Andes to Tropical Mexico is continuously high in fern species richness at our 1°-scale. We suspect that there may be narrow regions of lower diversity occurring along this length. For example, the lowland tropics of the Nicaraguan depression have been noted previously as a low-diversity area for ferns that separates the Mexican-Guatemalan highlands from Costa Rica and Panama (Moran, 1995). Because of their relatively large size, our 1°×1° grid cells may be too large to detect these patterns. We do, however, recover areas of lower richness and endemism in the regions between the Tropical Andes, the Guiana Highlands, and Southeastern Brazil. These areas are all separated by large tracts of tropical lowland forest, albeit with varying ecosystems. Related to this, our results show that the lowland tropical rainforests in the Amazon, known for its exceptional species richness in woody trees (Cardoso *et al.*, 2017), is a depauperate site of fern species richness (Fig. 1a; Fig. 3b), perhaps due to its relatively uniform topography and climate.

Richness.—Each fern hotspot circumscribed here is principally montane and our results clearly indicate a mid- to upper-elevational peak in species richness (Fig. 3b). This is a well-documented pattern within ferns and other lineages of tropical plants and animals (Rahbek, 1995; Bhattarai *et al.*, 2004; Cardelús *et al.*, 2006; Watkins *et al.*, 2006; Kessler *et al.*, 2011; Salazar *et al.*, 2015; Quintero and Jetz, 2018). Determining why ferns adhere to these patterns is important in explaining the distribution of neotropical fern diversity. One explanation may come from the mid-domain effect (MDE) (Watkins *et al.*, 2006), which states that species richness is generally a product of geometric constraints of range sizes (Colwell and Lees, 2000). Others have argued that the importance of MDE has been overstated, and fern diversity may be better explained by climatic factors (Kessler *et al.*, 2011). For example, in Central America nearly ever-humid conditions exist around ~2000 m in elevation and this creates a high diversity of epiphytic niche space (Vogelmann, 1973; Kluge, Kessler, and Dunn, 2006; Karger *et al.*, 2015). As proposed for ferns on a global scale (Suissa, Sundue, and Testo in review), we argue that the mid- to upper-elevational peak in neotropical fern species richness is amplified by the success of epiphytes in these ever-humid environments (Küper *et al.*, 2004; Cardelús *et al.*, 2006). This is supported by the heightened diversity of epiphytic ferns, as well as other lineages of vascular epiphytes in cloud forests (Gentry and Dodson, 1987; Cardelús *et al.*, 2006; Watkins *et al.*, 2006). In fact, some of the most diverse clades of ferns in the neotropics are predominantly epiphytic, such as *Asplenium* L., *Elaphoglossum* Schott ex J. Sm., Hymenophyllaceae, Polypodiaceae (Schuettpelz and Pryer, 2009; Testo and Sundue, 2016; 2018).

Diversification.—We find that circumscribed hotspots harbor more rapidly radiating lineages compared to surrounding regions. We further see that available niche space is significantly higher in these sites compared to surrounding regions (Fig. 5a inset) and that there is a strong positive relationship between niche heterogeneity and average speciation rates of lineage assemblages within hotspots (Fig. 5a). The Tropical Andes exemplify this pattern most strongly, as we recover some of the most rapidly radiating lineages in this site (Fig. S4b; Fig. S1b). The Andes have been identified as harboring rapidly radiating lineages in other vascular plants, such as club mosses (Testo, Sessa, and Barrington, 2019), bellflowers (Lagomarsino *et al.*, 2016), lupins (Hughes and Eastwood, 2006), and orchids (Pérez-Escobar *et al.*, 2017). These studies have attributed *in situ* diversification in the Andes to the cycles of uplift over the last 30 million years, which have led to the formation of new habitats such as high elevation alpine zones (Gregory-Wodzicki, 2000; Garzione *et al.*, 2008). While the Andes are one of the most notable examples of recent orogeny, there are other young mountain ranges scattered throughout Mesoamerica, Tropical Mexico (Dengo *et al.*, 1970; Janzen, 2018), and the Greater Antilles (Khudoley and Meyerhoff, 1971), where we also recover a strong relationship between niche heterogeneity and speciation rates (Fig. 5a). Steep elevational clines in young tropical mountains have layers of stacked habitats in a relatively small geographic space (Janzen, 1967; Hoorn *et al.*, 2010; Perrigo *et al.*, 2020). We propose that narrow climatic zones and seasonal stability within tropical mountains leads to lower physiological tolerance of fern species, which eventually leads to decreased range sizes, dispersal, and gene flow, all promoting *in situ* speciation (Janzen, 1967; Barrington, 1993; Kluge, Kessler, and Dunn, 2006; Polato *et al.*, 2018).

In addition to high niche heterogeneity within steep montane regions, it has been suggested that the scattered distribution of cloud forests and disjunct alpine zones from the northern Andes to Tropical Mexico act as "sky-islands" allowing discrete populations to inhabit the different peaks throughout the region (Bagley and Johnson, 2014). Cycles of climatic drying throughout the Miocene (Zachos, Dickens, and Zeebe, 2008) may have led to range expansions and contractions, leading to occasional introgression followed by reduced gene flow between populations (Bagley and Johnson, 2014). These further add to the diversification processes leading to the observed patterns of mountain biodiversity.

However, not all hotspots recovered in our analysis have young mountain ranges. Within the Guiana Highlands the sandstone plateaus are composed of rocks with sediments on the order of 1-billion years old (Huber, Prance, and Kroonenberg, 2018). Tectonic uplift within this region, which started ~90Ma in the Mesozoic (Kroonenberg and de Roever, 2009), is still continuing but much less rapidly than other neotropical mountains such as the Andes (Huber, Prance, and Kroonenberg, 2018). There are many other ecological and geological features of the Guiana Highlands that distinguish it from other parts of the neotropics, including white-sand soils, extensive river systems, unique rainfall regimes, and the tepuis (< 3000 m) (Huber, Prance, and Kroonenberg, 2018). Erosion and chemical dissolution of the tepuis led to the formation of a highly fragmented landscape (Briceño and Schubert, 1990; Schubert, 1995; Edmond *et al.*, 1995; Doerr, 1999). In Rapateaceae, Givnish *et al.* (2000), found that this fragmentation may have helped isolate populations on different mountain tops leading to reduced gene flow and allopatric speciation. Examination of our data from the Guiana Highlands suggests that *Lindsaea* Dryand. ex Sm. is the most rapidly radiating lineage and allopatry through the development of fragmented tepuis may be driving these speciation patterns.

Synthesizing our results from across hotspots, we conclude that the presence of rapidly radiating lineages (Fig. S4; Fig. S1b; Fig. S3b), high topographic and niche availability (Fig. 5; Fig. S3a), and their correlation within hotspots (Fig. 5a), suggest that habitat heterogeneity in montane regions as well as the presence of sky-islands combined with climatic fluctuations on geologic time-scales, are important in fostering fern diversity in the neotropics. These patterns are consistent with a process of *in situ* diversification explaining the exceptional richness of neotropical fern diversity (Fig. S4b; Fig. 5b). However, migration can also affect these patterns. For instance, average speciation rates of lineage assemblages within a given grid cell may be confounded by species that diversified within one area and then migrated to another. This is probably a common occurrence in ferns and has the effect of increasing the average speciation rates of lineage assemblages in a grid cell in the absence of *in situ* diversification.

Southeastern Brazil may be a site where the combination of migration and the persistence of relictual endemics are responsible for shaping biodiversity patterns. In this hotspot we find a weak relationship between niche heterogeneity and speciation rates of lineage assemblages (Fig. 5a). We propose that this is due to its complex biogeographic history. As cycles of climatic drying occurred throughout the Miocene (Zachos, Dickens, and Zeebe, 2008), the mountains in Southeastern Brazil may have acted as climatically stable refugia for lineages to inhabit, which has been suggested in other vascular plants such as Gesneriaceae (Perret *et al.*, 2013). Furthermore, historical migration of species between Southeastern Brazil and the Tropical Andes allow lineages with different diversification patterns to mix together.

This is supported by the high percentage (~20%) of shared fern species between Southeastern Brazil and the Tropical Andes (Fig. 4a), as discussed by previous studies (da Silva, 1995; Windisch and Tryon, 2001; Kreier *et al.*, 2008). For example, within our dataset, *Jamesonia osteniana* (Dutra) G.J. Gastony occurs in the mountains of Southeastern Brazil along with *Jamesonia cheilanthoides* (Sw.) Christenh. The former is a Brazilian endemic exhibiting a low lineage specific speciation rate; the latter is a recent Brazilian migrant that originated in the Andes, exhibiting one of the fastest diversification rates in our data. We suspect that this complex biogeographic history explains the weak correlation between niche heterogeneity and lineage specific speciation rates in this hotspot.

Endemism.—Locally endemic species can arise through the persistence of relictual lineages (paleo-endemism) (Fischer, 1960; Stebbins, 1974; Gaston and Blackburn, 1996), or through narrowly restricted species from rapid radiations (neo-endemism) (Haffer, 1969; Richardson *et al.*, 2001). Within the neotropics, fern endemism peaks in the Tropical Andes, Greater Antilles, and Mesoamerica (Fig. 2b; Fig. S4d; Fig. S1d). The high average speciation rates of lineage assemblages and high endemism in the Tropical Andes (Fig. S4) suggest the presence of neo-endemic species. These patterns are corroborated on a global (Suissa, Sundue, and Testo in review) and regional scale (Sánchez-Baracaldo and Thomas, 2014). Moreover, an examination of our data suggests that some individual Andean endemics have high lineage specific speciation rates such as *Serpocaulon patentissimum* (Mett. ex Kuhn) A.R. Sm. and *Elaphoglossum orbignyanum* (Fée) T. Moore. In contrast within the Greater Antilles, we see similar levels of endemism but some of the lowest speciation rates of lineage assemblages (Fig. S4). The Greater Antilles has been demonstrated to be a site of mixed paleo- and neo-endemism (Suissa, Sundue, and Testo in review), and is known to be a site of exceptional endemism for ferns (Maxon, 1922), as well as other vascular plant lineages (Santiago-Valentin and Olmstead, 2004; Nieto-Blázquez, Antonelli, and Roncal, 2017). We think that the Greater Antilles may represent a site with a mix of neo-endemics derived from *in situ* diversification as well as the persistence of relictual paleo-endemics buffered from extinction within the insular island chain. This is exemplified in our dataset by species such as *Mycopteris cretata* (Maxon) Sundue that we interpret as neo-endemic and species such as *Odontosoria jenmanii* Maxon and *O. scandens* (Desv.) C. Chr. that we interpret as paleo-endemic.

Distribution patterns.—By investigating the relationship between shared species, distance, and elevation throughout the neotropics we were able to quantify the patterns of fern community assemblages at regional scales. We found that both distance and elevational differences between sites are important in determining species composition (Fig. 4b, S2). Within ~1600 km, species similarity between any two grid cells is high; at the same time, grid cells with large elevational differences have low species similarity, even if they are geographically in close proximity (Fig. 4b; Fig. S2). This suggests that elevational differences determine species assemblages more strongly than geographical distances between sites. The effect of elevation and geographical distances (including latitude) on floristic composition has been understood since Humboldt clarified it in *Géographie des Plantes* (1805), but to our knowledge, explicit quantifications of these patterns have been lacking in ferns on a large scale.

At a distance of ~1600 km between any two grid cells, our results indicate a point of inflection where the curve flattens asymptotically. We suspect that sites further than ~1600 km apart are likely to only share widespread species. Tryon (1970) suggested that spore dispersal is common within ~800 km but tapers off strongly after ~1600 km. We agree that limits to spore dispersal play an important role in species range sizes and the point of inflection recovered at ~1600 km may relate to that (Fig. 4b). The microscopic wind-dispersed spores of ferns are known to travel great distances (Tryon, 1970; Page, 2002; Wild and Gagnon, 2005), with some known disjuncts from long-distance dispersal as far as the Andes to Madagascar (Bauret *et al.*, 2017). Other factors controlling the patterns of species assemblages may also be associated with distance, such as geographic turnover of niche space, physical geography, and the clade age of a lineage. For instance, if successful spore dispersal is a product of time (among other aspects of biology), then older clades would be more likely to have wider distributions compared to younger ones.

The average proportion of shared species remains relatively constant for a large distance between ~2000 and ~5000 km (Fig. 4b). This may illustrate the distribution of a number of widespread species (*e.g.*, *Asplenium fragrans* Sw. and *Botrypus virginianus* (L.) Michx.) that occur from Tropical Mexico to Brazil. These species may have diverged from the usual pattern of distribution, which could be associated with morphological, physiological, or reproductive traits. At ~8000 km similarity between sites approaches zero. This might be interpreted as the limit of spore dispersal, but more likely it is the limit of geography in the neotropics, as the furthest point between northern and southern tropical America is ~8000 km. Furthermore, the low shared species between sites at elevational differences greater than ~1000 m (even at relatively close geographic distances) suggests that, despite the ease of spore dispersal, ferns demonstrate strong niche conservatism across elevation transects (Watkins *et al.*, 2006). This further supports the hypotheses that ferns are diversifying in topographically complex and ecologically diverse sites with low seasonality montane ecosystems in the neotropics (Tryon, 1972; Barrington, 1993).

ONLINE ONLY SUPPLEMENTARY FIGURES

FIG. S1. Boxplot comparisons of values for a. Phylogenetic diversity, b. log (speciation rates), c. Phylogenetic endemism, and d. Weighted endemism, in 1°×1° grid cell in all hotspots. Points indicate values for individual grid cells. Boxes indicate interquartile range of values, darker line in box indicates mediate values, whiskers indicate 1.5× interquartile range. Brackets with '*', '**', or '***' indicate significant differences ($p < 0.05$, $p < 0.01$, or $p < 0.001$), NS indicates that the means are not significantly different.

FIG. S2. Three-dimensional plot depicting the relationship between elevation and distance of any two 1°×1° grid cells across the neotropics. Each point is a comparison between any two grid cells. Points are colored by the percent of shared species between the two grid cells.

FIG. S3. a. Boxplots of a. topographic heterogeneity and b. log (speciation rates), per 1°×1° grid cell in all hotspots. Points indicate values for individual grid cells. Boxes indicate interquartile range of values, darker line in box indicates mediate values, whiskers indicate 1.5× interquartile range. Brackets with '*' indicate significant differences (p, < 0.05).

FIG. S4. a. Phylogenetic diversity, b. mean log (speciation rate) of lineage assemblages, c. Phylogenetic endemism, d. Weighted endemism within 1°×1° grid cells. Colored cells indicate the estimated values for each 1°×1° grid cell.

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The Biogeography of Polyploid Ferns Across Space and Time

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Abstract.—A review of work on the origin and phylogenetic relationships of six systems of allopolyploid homosporous ferns and their progenitors serves as the basis for assessing the history and status of ideas relating to the biogeography of polyploid ferns. The likely dates of origin for the polyploids from recent analyses provide a historical context not previously available, making possible rigorous tests of hypotheses about the history of polyploids, especially the origin, distribution, and duration of polyploid ferns as lineages. Insularity figures prominently in developing an understanding of polyploid fern biogeography. The origin and persistence of polyploid ferns is enhanced by the empty niche space and high disturbance on Darwinian islands. In addition, the greater precinctiveness of polyploid ferns related to larger spore size is likely a selective advantage on islands, consistent with the number of high polyploids encountered among ferns on oceanic islands. Though recently originated polyploid ferns usually have identifiable progenitors with which they are partially sympatric, the rate of expansion of these ferns beyond the range of their progenitors varies. In two cases from Austral regions, long-term persistence and divergent speciation have been demonstrated in widespread polyploids and polyploid lineages with unknown diploid progenitors, though the general consensus is that polyploids do not persist in the long term.

Key Words.—allopolyploidy, historical biogeography, ferns, dated phylogeny, spore size

Historical biogeography as a discipline has undergone dramatic change in the last 60 years. The current approach has at its center inferring the geographic history of living things with vicariance as a null hypothesis, to be rejected before dispersal hypotheses are proposed (Brundin, 1966; Agnarsson, Ali, and Barrington, 2019). However, in the last 20 years, a consciousness of the ecological factors that determine the distribution of living things has been restored to historical biogeography (Wiens and Donoghue, 2004)—factors that have in fact been inherent in the discipline since its origin (von Humboldt and Bonpland, 1807). Analyses now include a growing awareness of and inquiry into the change in these factors over time (Matzke, 2013). Though these parameters are all now part of the biogeographer's perspective, polyploids remain outliers to be extracted from consideration so that the historical biogeography of divergent species is possible to infer. This contribution is an exploration of the historical biogeography of allopolyploid ferns from such an integrated perspective.

Oceanic islands are central to any general inquiry into historical biogeography. A number of characteristics of these islands are directly relevant in general and important in considering allopolyploids in particular. First, oceanic islands have never been in direct geographic contact with a continental source of colonists and as a result have abundant empty ecological niche space (Gillespie, Claridge, and Roderick, 2008). Successful colonization is made more likely for arriving propagules because oceanic islands provide this empty niche space, and the characteristic geographical isolation of these islands then provides the opportunity for speciation (MacArthur and Wilson, 1967; Whittaker *et al.*, 2008). However, persistence and diversification are tempered by extinction, which has a prominent (and presumably more significant) role on islands relative to continents (MacArthur and Wilson, 1967). Second, oceanic islands are inherently transient, because they have a typical life cycle comprising origin via vulcanism followed by subsidence and loss via erosion with the cessation of volcanic activity (Darwin, 1889; Whittaker *et al.*, 2008); thus no one island provides a long-term residence for a species, diploid or polyploid. Third, the characteristic volcanic activity and erosional subsidence of these islands leads to high levels of ecological disturbance in their plant and animal communities.

In addition to these observations about the ecology and geology of islands, there is a relevant pattern to the size of plant propagules on islands, regardless of evolutionary lineage: they are often larger than those of their continental allies. The cause of this pattern is taken to be selection for greater fitness following from propagule concentration in the island communities rather than loss to the surrounding ocean, an idea known as precinctiveness (Carlquist, 1966). Since volumes of tetraploid ferns are commonly double that of the combined volumes of their progenitors (Barrington, Patel, and Southgate, 2020), the polyploids are more precinctive, as evidenced by work of Raynor *et al.* (1976) documenting the lower dispersal of larger *Osmunda* spores relative to smaller *Dryopteris* spores into a forest.

Finally, Gillespie and Roderick (2002) have distinguished *Darwinian islands*, defined as newly created habitat originating within a matrix of unlike habitat, from *fragment islands*, defined as habitat isolated from a larger habitat by expansion of a matrix of unlike habitat. Darwinian islands thus include not only oceanic islands but habitat islands on continents. Important for the goals of this contribution are two specific kinds of habitat islands: substrate islands (Kruckeberg, 1991) and montane (sky) islands (Warshall, 1995).

Previous work on the historical biogeography of polyploids in the ferns is grounded in two mid-twentieth-century works, those of Irene Manton and her Ph.D. student John Donald Lovis. Manton (1950), in the general conclusions to her extraordinary compendium of studies on polyploidy in Europe, sums up her thoughts on the geographic patterns of polyploids espoused by European authors working on angiosperms with her assessment of the patterns in ferns, including reference to her own work on the ferns of Madeira, then in progress. She begins with her impression that in Europe there has been a recent *epidemic* of polyploidy in ferns as in angiosperms, leading to the extinction of some progenitors with the expansion of their allopolyploid derivatives. Looking to cause, she rejects polyploidy as adaptive to a particular physiological variable (specifically cold) for its being favored by dramatic environmental change, *i.e.,* the climatic upheaval of the Pleistocene in Europe. In Manton's view, hybridization, and thus the origin of allopolyploids, are enhanced by an array of perturbations in climate or topography, including changes in temperature or precipitation and mountain building; notably she includes volcanic action. Turning to her work on Madeira, there she noted the unusual number of polyploids above the tetraploid level, all of which were without extant relatives on the islands (or elsewhere), and suggested that they are witnesses to an episode, earlier than the Pleistocene, of disturbance resulting in hybridization. She also concluded that polyploids (*i.e.*, neopolyploids) have limited long-term evolutionary potential, noting that unlike the products of divergent evolution polyploids are single species, not lineages of species.

Lovis (1977) affirmed Manton's ideas about high polyploidy on Madeira almost three decades later in his articulate summary of the work to that date on patterns in the geographic distribution of polyploid ferns. He also provided evidence that two allotetraploids in the Hymenophyllaceae and an incipient hexaploid species in the *Asplenium trichomanes* complex had originated recently on the island. He suggested that the insular origin and persistence of these endemic, recently originated Madeira polyploids are due to the reduced competition in the low-diversity oceanic-island ecosystem relative to the more complex structure of continental communities. In this way, he applied the open-niche idea later articulated by Gillespie, Claridge, and Roderick (2008) to polyploid ferns on islands.

In a number of respects, Stebbins—writing in North America—anticipated Manton's ideas in his angiosperm-oriented paper on the evolutionary significance of polyploids (Stebbins, 1940). He contended that polyploid plants are dominant in recently opened regions and regions that are undergoing great climatic or other environmental changes. In contrast, he saw the diploids as tending to occupy older, more stable *(i.e.,* less dominated by disturbance) habitats. His observations led him to conclude that the polyploids are dominant in regions recently opened to occupation by plants or subjected to great climatic or other environmental changes. Stebbins's evaluation of this pattern is interesting: "This makes the study of polyploid complexes very important from the standpoint of plant geography." Considering the two writers together, the message is that the disturbance that brings species together, creating the opportunity for hybridization, yields the hybrids with the potential to become allopolyploids. Consequently, the geographical distribution of polyploids reflects the distribution of disturbance across the earth.

Although the geographic distribution of polyploidy in the ferns has received little or no attention in recent years, angiosperm researchers continue to be impressed by the number of polyploids at higher latitudes and altitudes, especially in Arctic regions (Soltis, Soltis, and Tate, 2004). The pattern may well be related to life form, since in angiosperms polyploidy is much more common in herbaceous perennials than in annuals or trees (Soltis, Soltis, and Tate, 2004). To fern biologists, whose plants are almost all herbaceous perennials, the increased frequency of this life form as an explanation for the higher frequency of polyploids in the Arctic has little explanatory power.

Given their dominant life-form, Lovis's rejection of a relationship between higher latitude and higher frequency of polyploid ferns makes sense. The virtual absence of polyploidy from the arborescent Cyatheaceae (Walker, 1984) is consistent with this idea.

Stebbins (1940) provided a working hypothesis for the historical biogeography of polyploids, again angiosperm-centered. Having observed that allopolyploids generally have wider geographical ranges than their diploid progenitors, he suggested that the progenitors would be the first to go extinct. In the end, in Stebbins's view, the polyploids would also go extinct, so that the entire complex reaches an end stage with only relictual polyploids. Manton's interpretation of the significance of the high-polyploids on Madeira, in contrast to those of Europe, is grounded in the same idea of polyploids having a life cycle. Corroboration of this hypothesis would take the form of restricted distributions and sympatry with known progenitors for newer polyploids, but broad distributions and missing progenitors for older polyploids. Comparative analysis of fern polyploids remains to be explored in the context of Stebbins's and Manton's ideas.

Recent analyses of angiosperms support ideas about the lack of long-term durability of polyploids, at least neopolyploids. Mayrose *et al.* (2011) analyzed a set of diploid and polyploid congeners including both angiosperms and spore-dispersed vascular plants, reaching the conclusion that polyploids diversify more slowly and go extinct more frequently than their diploid allies. Though this conclusion has been questioned (Soltis *et al.*, 2014), the overall pattern of shared polyploid chromosome number characterizing species and small, recently diverged clades seems like convincing evidence that polyploids, at least neopolyploids, have not persisted in the long-term, even if in the short term they may overwhelm their progenitors. Ironically, whole-genome duplication, ordinarily as the result of polyploidy, has apparently had a major role in the macroevolution of major plant lineages (Clark and Donoghue, 2018).

Is the geographic range of a polyploid fern related to its age? Do progenitors indeed go extinct as their polyploid progeny expand over time? Do islands, oceanic or ecological, provide the basis for insights into the biogeography of polyploids in the same way they have for the historical biogeography of divergent species (Tryon, 1970)? In this contribution I consider the historical biogeography of polyploid ferns in the context of the Lovis and Manton-Stebbins ideas from a review of the work on six different systems in the Barrington lab at the University of Vermont over the years between 1983 and 2020. The early work used approaches developed in the 1950s and 1960s to explore the constitution of polyploids; the more recent work has added the molecular-genetic techniques as they have become available. These projects, taken together, yield a broad array of biogeographic patterns to be found in polyploid ferns and their progenitors (where they are known).

All of the projects specifically address polyploid complexes whose polyploids are sexually reproducing and originate in hybridization (*i.e.,* allopolyploids), limiting the inquiry here to a smaller field in which inferences are more likely to withstand scrutiny. Advances in our ability to infer ancestry and to delimit the likely ages of origins in each of the systems (Table 1) in the context of improved geological insights into Cenozoic history inform the inquiry. Insights from consideration of ecological and evolutionary parameters specific to ferns, especially in reference to Darwinian islands, add to our understanding of the historical biogeography of these six sexual allopolyploid lineages. Three different projects we have addressed in our lab address complexes from Darwinian islands: one on an oceanic island (*Polystichum* on the Hawaiian archipelago) and two on recently originated habitat islands (*Adiantum* on serpentine in the northeastern North America and *Polystichum* in the tropical Andes páramos). Four projects addressing recent origins contrast with two projects addressing older origins.

ADIANTUM VIRIDIMONTANUM

As a guest researcher in Chris Haufler's lab at the University of Kansas, Cathy Paris, using the then novel technique of isozyme analysis, discovered that Vermont plants of *Adiantum* growing on serpentine substrates included allotetraploids. She was able to demonstrate that the plants, described as *Adiantum viridimontanum* C. A. Paris, combined the heritage of two diploid species, *A. pedatum* L. and *A. aleuticum* (Rupr.) C. A. Paris. Cathy also demonstrated that *A. aleuticum* and *A. pedatum* are in general obligate outcrossing diploids, and she documented the disjunct distribution of *A. aleuticum* between the serpentine sites in northeastern North America and sites on a broader array of substrates in western North America extending into the Aleutian archipelago (Paris and Windham, 1988; Paris, 1991). These observations led her to the inference that the disjunct northeast populations of *A. aleuticum* were there as the result of vicariance. She suggested that *A. aleuticum* had expanded into a broad-ranging periglacial distribution across North America in the late Pleistocene, the expansion idea originating with Fernald (1925). More recently, as the periglacial habitats have disappeared with the advent of warmer climate in the Holocene, the eastern populations of *A. aleuticum* persisted only in the low-competition community on serpentine-bedrock substrate that many other plants could not occupy (Paris and Windham, 1988).

Lu *et al.* (2011) generated a chloroplast-DNA dated phylogeny of the tetraploid and its progenitors in the context of their eastern allies, yielding a timetable for the origin of the northeast North American *A. viridimontanum*—less than 1 million year ago (mya), see Table 1. The continental glaciers covering all of the present-day distribution of the tetraploid suggest that its origin is much younger, within the last 12,000 years.

More recently, Morgan Southgate, a Ph.D. candidate in our lab, characterized the population-level ecology of the polyploid and its progenitors in order to understand the ecological conditions in which polyploids originate at the local level. She characterized the ecological niches of the three eastern North American taxa, revealing that the serpentine terrain shared by diploid *A.*

TABLE 1. History, geography, progenitors where known, and age calibration for the systems in this review

Species	History and relative endemicity	Geography	Progenitors where known	Age of origin	Age basis
Adiantum viridimontanum	recent origin, restricted distribution	North-temperate edaphic island	*A. pedatum* × *A. aleuticum*	<1 mya	chronogram, Lu *et al.*, 2011
Polystichum páramo tetraploid	recent origin, broader distribution with disjunct populations	Tropical montane island	*P. polyphyllum* × *P. sodiroi*	<1.4 mya	divergence from *P. polyphyllum*—chronogram, McHenry & Barrington, 2014
Polystichum talamancanum	recent origin, restricted distribution	Tropical montane island	*P. concinnum* × *P. polyphyllum*	<1.8mya/ <2.7mya	divergence from *P. concinnum*—chronogram, McHenry & Barrington, 2014/ ditto Morero *et al.* 2019
Polystichum lilianiae	recent origin, restricted distribution	Tropical montane island	*P. concinnum* × *P. turrialbae*	<4.3 mya	*concinnum* origin—chronogram, McHenry and Barrington, 2014
Polystichum californicum	relatively recent origin, distribution somewhat surpasses progenitors	Temperate coastal forests, widespread	*P. dudleyi* × *P. imbricans* subsp. *curtum*	<6 mya[1]	*P. munitum* origin—chronogram, Morero *et al.*, 2019
Polystichum haleakalense and *P. bonseyi*	recent origin, restricted distribution	Tropical montane forest on oceanic archipelago	*P. haleakalense* is one progenitor of *P. bonseyi*	< 5 mya	earliest available elevation for the appropriate habitat
Polystichum braunii	earlier origin, broad distribution	Cool-temperate to boreal broadleaf forests, disjunct circumboreal	unknown	<7.7 & <10.0 mya[2]	chronogram—Jorgensen and Barrington, 2017
Austral South American clade	earlier origin, broad distribution	Cool South-temperate (4x clade is circum-austral)	unknown	<7.9 mya[3]	chronogram—Morero *et al.*, 2019

[1] for the origin of the *P. munitum*-*P. imbricans* clade

[2] origin of the western China and widespread haplotypes respectively

[3] origin of the austral South American clade; including the Australasian species (all polyploid), the origin of the polyploid lineage is 10.6–15.0 mya.

aleuticum and tetraploid *A. viridimontanum* is in fact partitioned, with the diploid more closely confined to niches in the intact ultramafic substrate (Southgate, Patel, and Barrington, 2019). The broader niche specificity of the polyploid, including sites that are more disturbed than those of either progenitor, aligns with the ideas of Stebbins and Manton about polyploids originating and persisting in disturbed habitats.

The relative size of spores in the *Adiantum aleuticum* complex has twice been included in our work on the utility of spore size in interpreting evolutionary history in the ferns (Barrington, Paris, and Ranker, 1986; Barrington, Patel, and Southgate, 2020). Two patterns have emerged, both significant. First, the volume of the tetraploid's spores is somewhat more than twice that of either progenitor's spores. Second, the volumes of the two diploids were significantly different, with the mean volume of spores from the serpentine-endemic populations of *A. aleuticum* in the northeast almost 40% larger than those of forest-dwelling *A. pedatum*. Thus, not only is the spore size of the tetraploid on the serpentine-substrate habitat island larger than the diploids, but the size of the diploid on the serpentine is larger that of the diploid in the surrounding forest matrix.

POLYSTICHUM CALIFORNICUM

Herb Wagner provided the first insights into a remarkable allotetraploid from the western North American cordillera, *Polystichum californicum* (D. C. Eaton) Diels (Wagner, 1963; 1973) from his work with mixed populations of the tetraploid with its progenitors, the once-pinnate *Polystichum munitum* (Kaulf.) C. Presl *s.l.* and twice-pinnate *P. dudleyi* Maxon, from Napa Valley canyons in California. His careful cytological and morphological analyses documented back-cross hybrids to the progenitors and, significantly, sterile diploid hybrids of the two progenitors, *i.e.,* new candidates for allopolyploid speciation. He writes, "What is remarkable is that [the] *de novo* diploid hybrids co-exist with derivative allotetraploids side-by-side in the same habitats, a state of affairs that to my remembrance has not previously been reported." Wagner at the time pointed out the need for a closer analysis of the variation in the once-pinnate *P. munitum* including what is now included in *P. imbricans* (D. C. Eaton) D. H. Wagner.

The heritage of *Polystichum californicum* has subsequently been complicated by the resolution of three different taxa within *P. munitum s.l.*: *P. munitum s.s.* and a second species, *P. imbricans*, comprising two subspecies, subsp. *imbricans* and subsp. *curtum* (Ewan) D. H. Wagner (Wagner, 1979). Significantly, these three taxa have different habitat preferences and somewhat differentiated geographic distributions, with *P. munitum* and *P. imbricans* distributed broadly from southernmost California to British Columbia and Montana but *P. imbricans* subsp. *curtum* distributed more locally from southern California to southwestern Oregon. Ecologically, *P. munitum* is found in stable habitats on the shaded floors of mesic forests, whereas both *P.*

imbricans subspecies are found on more disturbed habitats on rock outcrops and rocky soils in drier forests.

Undergraduate Melita Schmeckpeper and I assembled a substantial set of western North American plants comprising all of these entities, thanks to field work carried out by a number of friends in the west. We sought to 1) test David Wagner's 1979 taxonomy of *Polystichum munitum s.l.* with a chloroplast DNA phylogeny combining the *rbcL* and *trnL-F* markers and 2) document the roles of the three taxa in the *P. munitum* aggregate in the origin of *P. californicum* using the nuclear marker *pgiC*. Melita's work, based on the chloroplast and nuclear DNA markers along with data from flow cytometry, spores, and morphometric analysis of fronds, provides a portrait of the biogeography and ecology of a nascent allopolyploid (Schmeckpeper, 2016).

Plants of the once-pinnate diploid comprised three clades in the cpDNA analysis; their phylogenetic relationships were congruent with Wagner's 1979 taxonomy recognizing three taxa in two species. Allotetraploids (documented by flow cytometry) all had as their once-pinnate progenitor *P. imbricans* subsp. *curtum*. However, diploid hybrids were of two types, incorporating *P. dudleyi* with either *P. munitum* or *P. imbricans* subsp. *curtum*. In addition, Melita documented the backcross of *P. californicum* (incorporating the *curtum* genome) to *P. dudleyi*. In sum, hybridization is continuing to yield candidate polyploids in a system where the resulting fertile tetraploid has expanded beyond the range of both of its progenitors.

Polystichum Polyploids in Tropical America

Inquiry into polyploidy in tropical American *Polystichum* spans the entire history of the Barrington lab at the University of Vermont. Our changes in methodology and datasets read like a history of plant systematics inquiry over the last forty years. For much of that time, our work on *Polystichum* has focused on the higher elevations of a single mountain, the Cerro de la Muerte, in the Cordillera Talamanca of southern Central America. Morphology, spore size, meiotic chromosome number and behavior, isozymes, chloroplast DNA, and nuclear DNA (direct and cloned sequences) have all provided witness to the history of these plants. One way or another, virtually every student who has spent time in the Barrington lab since 1980 has contributed to the effort. In recent years, having documented three tetraploids on Cerro de la Muerte (all, as it turns out, new to science), we have broadened our focus to include the northern and central Andes, where the situation is more complex. The work on the Cerro de la Muerte, based on repeated study over many years and an array of techniques, has equipped us for the daunting task of untangling the Gordian knot to the south.

The three tetraploids are all twice-pinnate allotetraploids; the first two have been described. *Polystichum lilianiae* Barrington is a small (*ca.* 0.7 m) species of the high-montane oak forests (*ca.* 3000 m) with very large indusia (for *Polystichum*, *ca.* 1.0 mm diam.). *Polystichum talamancanum* Barrington is a larger species of shrubby páramos and dwarf forests (*ca.* 3200 m) dominated by

Escallonia myrtilloides L. f., *Buddleia nitida* Benth. in DC, and *Solanum pulverulentum* Pers.; it is exindusiate except for a few sori with vestigial indusia near the rachis. The undescribed *Polystichum* tetraploid (hereafter the *páramo tetraploid*) is from the open páramos (3400 m and higher); it has no indusia and is unique among the three in having one or two pinnatisect pinnules at the base of the pinnae and virtually lacking spinule development on the frond.

Early cytological and isozyme work with species and their hybrids on the Cerro de la Muerte revealed that *Polystichum talamancanum* has as one of its progenitors *P. concinnum* Lellinger ex Barrington, a very large (fronds to 2 m) indusiate species endemic to the oak forests of the Cordillera Talamanca (Barrington, 1990). Chloroplast DNA sequences corroborated *P. concinnum* as a progenitor (maternal) and revealed that the phylogenetic affinities of *P. concinnum* are with a clade of indusiate tropical American species most diverse in southern Mexico and Guatemala (Driscoll and Barrington, 2007). The isozyme and chromosome work also documented the existence of a second progenitor, shared by *P. talamancanum* and the páramo tetraploid (Barrington, 1990), which has not been discovered on the Cordillera Talamanca.

The search for the missing diploid progenitor shared by two Cerro tetraploids has been prominent in my research agenda for the past thirty years. Morphologically, the páramo tetraploid resembles an array of Andean páramo ferns, the spore sizes of which early suggested that both diploids and tetraploids were among collections from the Andes (Barrington, Paris, and Ranker, 1986). Stacy Jorgensen, working with me for her undergraduate thesis in 2009, approached the problem by cloning the nuclear marker *gapCp* using a sample of the tropical American *Polystichum* taxa then available. Though support values were low, one of the two páramo tetraploid clones resolved with an accession of the diploid Andean endemic *Polystichum sodiroi* Christ and the other with Andean *P. paramicola* Kessler and A. R. Sm., both páramo species. In 2008, doctoral candidate Monique McHenry took on a broad phylogenetic analysis of the tropical Andean *Polystichum* species. Her extensive Andean field work over four trips in 2010 and 2011 provided us with the broad sample needed to explore the origins of Andean species. Monique's chloroplast DNA phylogeny (McHenry and Barrington, 2014) of the tropical Andean lineage confirmed the existence of an Andean clade of exindusiate species first discerned in earlier work in our lab by master's student Heather Driscoll (Driscoll and Barrington, 2007). Monique provided substantial resolution within the Andean clade, leading to a historical biogeography of the clade concordant with the rise of the central and more recently northern Andean mountain ranges. In this work, accessions of the páramo tetraploid were nested within a clade that comprised four high-elevation taxa often included in *P. orbiculatum* in floristic works but which she recognized as species, including *P. polyphyllum* (C. Presl) C. Presl and *P. sodiroi* Christ as well as *P. orbiculatum*. Geological constraints date the origin of all the páramo taxa to within the last 5 mya.

University of Vermont undergraduate Brendan Lyons accepted the challenge of developing a nuclear marker to yield better resolution to the history of the Andean *Polystichum* lineage, focused on *pgiC*. Brendan's work yielded a profile for the gene in *Polystichum* based on sequences of about two-thirds of the gene. Plants determined as *P. orbiculatum* (from Bolivia) and *P. polyphyllum* (from Ecuador) were included in the dataset. They were not sister to one another, and the absence of double peaks in the *pgiC* chromatograms implied that the accessions of both were diploid and hence candidate progenitors for the páramo tetraploid (Lyons, McHenry, and Barrington, 2017).

Our search for the progenitors of the páramo tetraploid has finally been successful with close analysis of Andean and Costa Rican plants using cloned nuclear-DNA marker sequences for two markers (*pgiC* and *gapCp*), in the work begun in 2009 by Stacy Jorgensen as an undergraduate in our lab and first reported in this issue (Jorgensen and Barrington, 2020). Hybridization of tropical Andean *P. polyphyllum* and the northern Andean endemic species *P. sodiroi* now appears to have yielded the páramo tetraploid. This outcome is consistent with the results of the cpDNA phylogeny (McHenry and Barrington, 2014), in which the three resolved in a single clade. *Polystichum paramicola*, though related, is more remote.

The last of the Costa Rican tetraploids to be discovered, *Polystichum lilianiae*, has also presented challenges to discovering its progenitors. Early on, it was clear that the broad-ranging high-forest tropical American species *P. turrialbae* Christ was one (Barrington, 2003). Though found throughout the continental Neotropics, *P. turrialbae,* like *P. concinnum,* traces its relationships to the northernmost American tropics, with the clade of indusiate species most diverse in southern Mexico and Guatemala. Since *P. lilianiae* consistently has indusia with diameters near the maximum encountered in the American tropics, the second progenitor is also likely to have Mexican affinities and have well-developed indusia. Again, a well-supported hypothesis for the identity of this progenitor has awaited the work based on cloned nuclear-DNA marker sequences in the work reported in the contribution by Stacy Jorgensen (Jorgensen and Barrington, 2020). Just as with *P. talamancanum*, the endemic high-montane *P. concinnum* appears to be the contributor.

It is now possible to provide a portrait of the polyploids on a single montane island, the summit of Cerro de la Muerte in Costa Rica. There, three tetraploids are elevationally distributed, largely occupying different plant communities, and presenting three different origin histories. Two, *P. lilianiae* and *P. talamancanum*, are endemic to the Cordillera Talamanca, the montane archipelago to which the Cerro de la Muerte belongs. These two most likely originated on the Cordillera Talamanca because they share the local-endemic *Polystichum concinnum* as a progenitor. The oak-forest species (*P. lilianiae*) has as its second progenitor the diminutive montane-forest *P. turrialbae*. The dwarf-forest/shrub-páramo species has as its second progenitor (the one shared with the páramo tetraploid) the tropical Andean páramo species *P. polyphyllum*—which suggests that this diploid species was present on the

Sierra Talamanca at some time in the Pleistocene and may still be there, but is so far undiscovered.

In contrast to the other two polyploids, the páramo tetraploid—found at the highest elevations of the three in the open páramo—is widespread in tropical America, all the way from the northern limits of Central America at the Guatemala-Mexico border to the northern limits of the central Andes in Peru. Its two progenitors are both now found only in the Andean páramos. The first, *P. sodiroi,* is a 3-pinnate species restricted to the southern part of the northern Andes (largely from central to southern Colombia and northern Ecuador). The second (also a progenitor of *P. talamancanum*) is *P. polyphyllum*, a diminutive species widespread in the northern and central Andes. The páramo tetraploid seems likely to have originated in the northern Andes where *P. sodiroi* is endemic, then reached the Cerro de la Muerte by long-distance dispersal. All three polyploids are at least in part sympatric with their diploid progenitors. All three have originated in the recent past, more recently than about 4 mya, based on dated phylogenies (Table 1); their dates of origin, from oldest to youngest, correlate with increasing elevation, *i.e.*, with availability of the habitat.

HAWAIIAN *POLYSTICHUM*

Three species of *Polystichum* are known from the Hawaiian archipelago; all are endemic (Palmer, 2003; Driscoll and Barrington, 2007). Each has a different ploidy: *P. hillebrandii* Carr. in Seem. is diploid, *P. haleakalense* Brack. is tetraploid, and *P. bonseyi* W. H. Wagner & R. W. Hobdy is octoploid. The three are found in mesic forests near the boundary with the drier subalpine woodland/shrubland zone at upper elevations (1400–3200 m) on Maui and Hawai'i, the highest islands and the youngest geologically. Heather Driscoll, a master's student in our lab, working in coordination with Tom Ranker's group on the biogeography and evolution of Hawaiian pteridophytes, developed a robust genetic profile for the three species with two datasets, isozymes and chloroplast DNA sequences (*rbcL* and *trnL-F*), following her own field work in Hawaii.

The cpDNA analysis yielded insights into the evolutionary relationships and biogeography of the three. Diploid *Polystichum hillebrandii* resolved with high-elevation wet-forest species of the eastern Himalayas and Heng Duan Mountains of western China in *Polystichum* section *Duropolystichum* Fraser-Jenkins. The polyploids resolved with *P. sinense* (Christ) Christ (*Polystichum* ser. *Sinensia* H. S. Kung & Li Bing Zhang), a species from similar habitats in the same geographic region of Asia (and farther west, including India, Africa, and the Mascarenes). The isozyme analysis indicated that the polyploids did not include a genome from the diploid, but that they shared a genome with one another—however the octoploid had a genome absent from the tetraploid, suggesting that both are allopolyploids.

Phylogenetic and geological data provide the basis for understanding the history of the Hawaiian polyploids. All three of the extant Hawaiian

Polystichum species resolve in Sino-Himalayan lineages. Diploid *P. hillebrandii* resolves with a suite of morphologically similar species, none of which are sexual diploids (Patel, 2017), so is certainly an endemic species. The tetraploid resolves with and closely resembles the widespread *P. sinense* (Christ) Christ, which is also an allotetraploid; they may be conspecific. The octoploid is morphologically unique though it resolves with *P. haleakalense* and *P. sinense*. Morphological and isozyme differences point to its autochthonous origin from hybridization of *P. haleakalense* with an extinct tetraploid. These inferences require three different dispersal events to the archipelago from the Sino-Himalayan region. Between eight and five mya, volcanoes with elevations over 2000 m in the Hawaiian archipelago were absent, and volcanoes with elevations over 1000 m were repeatedly reduced down to one. Distance to the next oldest volcano with high elevation in the period increased to 150 km (Price and Clague, 2002). Thus, the high elevations suitable for the extant *Polystichum* species in the archipelago were probably absent until after re-initiation of active volcanic activity 5 mya. In addition, migration between islands of the archipelago became more unlikely. Taken together, these parameters suggest an earliest date of arrival of spores with *Polystichum* genomes in the archipelago of 5 mya, followed by the more recent polyploidy and extinction events.

POLYSTICHUM BRAUNII

Polystichum braunii (Spenner) Fée is tetraploid throughout its interrupted montane north-temperate range (Jorgensen and Barrington, 2017; Sleep, 1966). Using study of meiotic chromosome pairing behavior during meiosis in synthesized hybrids, an effective approach popular in European labs at the time, Manton and Reichstein (1961) demonstrated that the species is an allotetraploid. Anne Sleep, based on an array of hybrids she synthesized, found no appreciable chromosome pairing at meiosis in any of her hybrids of European or Japanese *Polystichum* species with *P. braunii* (Sleep, 1966). *Polystichum braunii*'s progenitors have remained undiscovered over the half century since Anne Sleep provided the first substantial insights into the species.

Stacy Jorgensen, going on to be a master's student working with me, used sequencing of chloroplast DNA and cloned nuclear DNA to provide insights into the history of the species and the relationships of its progenitors (Jorgensen and Barrington, 2017). Her combined analysis of two nuclear markers (*pgiC* and *gapCp*) revealed that *P. braunii* had the same two progenitor genomes throughout its broad range, but her chloroplast work (combining *rbcL, rps4-trnS*, and *trnL-F*) revealed two highly divergent haplotypes: *Polystichum braunii* has at least two origins.

Stacy included a broad array of North American and morphologically similar Asian species in her study set. Both the chloroplast and nuclear datasets resolved *Polystichum braunii* in two widely divergent clades, one North American and one eastern Asian. We interpreted this pattern as evidence that members of the two lineages had been sympatric at some point to make hybridization possible; our dated phylogeny allowed the inference that the date of contact was likely to have been Late Miocene (Table 1). The most likely place for that contact was in Beringia, which included extensive terrestrial habitat at the time. Mid- to late-Miocene fossil floras from the region (Wolfe, 1994) document forests suggesting a mean annual temperature and annual temperature-range parameters similar to those we retrieved for *P. braunii* using the mean and range of annual temperatures for the species we developed from the WorldClim database (Hijmans *et al.*, 2005).

Still, *Polystichum braunii*'s progenitors remain at large. Morphologically, *Polystichum braunii* is unlike any North American or European *Polystichum* species in its herbaceous rather than chartaceous to coriaceous texture and its frond much narrower at the base that at the middle. We included Asian species with these features in our dataset, but none resolves close enough to the *P. braunii* accessions in the nuclear DNA analysis to argue for their being progenitors; thus *P. braunii*'s progenitors are extinct to the best of our current knowledge. We retrieved remarkably little genetic differentiation between accessions of the species, suggesting that recent long-distance dispersal, rather than expansion following early after its origin, has established the present broad distribution of *P. braunii* with the North American progenitor's cpDNA across the north.

Austral South American *Polystichum*

Resolution of the austral South American species of *Polystichum* as a monophyletic lineage with a unique history has emerged from a set of contributions published over a century. Hermann Christ first drew attention to the importance of studying the species in the region while reporting on a set of plants received from the Skottsberg South-Polar Expedition of 1901–1903 (Christ, 1905). Christ included what we now know to be species from both Andean exindusiate and Austral lineages in his review of austral *Polystichum* species: among the indusiate taxa two species from austral South America were included. Looser (1968) provided the first taxonomic treatment of the group using species concepts largely like the current ones. Refinement of the taxonomy of the austral South American species came with Rodriguez's work in the 1980s and 1990s (Marticorena and Rodríguez, 1995; Rodriguez, 1987), yielding understanding of lineage diversity and biogeography (*i.e.,* including both the austral Andes and three archipelagos, the Juan Fernandez and Falklands (Malvinas) as well as South Georgia. (He, like Christ, included remotely related species from other Austral islands.)

Rita Morero of the Instituto Multidisciplinario de Biología Vegetal, Córdoba, Argentina, joined our lab as a guest scientist during 2012 with the goal of developing an understanding of the phylogeny and biogeography of the austral South American species of *Polystichum*. Her work, realized in a series of four publications (Morero *et al.*, 2015; Morero, 2016; Morero *et al.*, 2016; Morero *et al.*, 2019), yielded our present sound understanding of the group and its relationships. Austral South America and nearby islands host a total of seven species, five in the austral Andes, one in the Juan Fernandez Islands, and one in the Falklands and South Georgia. Rita's analysis of cpDNA sequences generated in our lab, plus her own cytological work yielding chromosome counts for all of the species in her study group, resulted in a phylogeny and historical biogeography for the lineage. The austral South American *Polystichum* species constitute all of the diversity in a monophyletic group that diverged less than 8 mya; it is sister to clades comprising all of the Australian and New Zealand species; together these lineages constitute a clade whose origin is Asian. Given the mid-Miocene divergence of the Austral clades (Table 1), long-distance dispersal of Old-World *Polystichum* to austral South America is the most likely history.

All of the austral South American *Polystichum* species are polyploid (comprising six tetraploids and one octoploid). The complete absence of diploids is also characteristic of the Australasian species; the most parsimonious explanation for this pattern is that their common ancestor was tetraploid, but at least in austral South America, nearly continuous morphological variation between species in the clade suggests a more recent history of reticulation—most recently yielding the octoploid *P. subintegerrimum* (Hook. & Arn.) R. Rodr.

SYNTHESIS

Insularity.—Insularity is at the heart of understanding the geography of polyploid ferns because islands present two key features; high levels of disturbance and empty niche space. On the Cerro de la Muerte Darwinian island, I early revealed a pattern of hybrid formation being enhanced by habitat disturbance. In an elevational framework, the progenitor species yielding *Polystichum concinnum* Lellinger ex Barrington and *Polystichum speciosissimum* (A. Braun ex Kunze) R. M. Tryon & A. F. Tryon were sympatric in disturbed terrain but not in intact plant communities (Barrington, 1985), consistent with the idea that hybridization is promoted in disturbed habitats. The hybrids I have documented on the Cerro de la Muerte (Barrington, 1990) are all from disturbed habitats, especially on roadcuts and in borrow pits. In the context of Pleistocene climate change associated with glaciation, the northeast North American, Costa Rican, and Andean habitat islands where we encountered our study groups with recently originated polyploids are all periglacial, and the periglacial environment is continuously shifting spatially (vertically in the tropics and horizontally in the North-Temperate) and thus chronically disturbed.

In the context of our modern perception of the origin and equilibrium of species diversity on islands (Gillespie and Roderick, 2002) just as for Lovis (1977), polyploid ferns are successful in originating and persisting on Darwinian islands because of the empty niche space there. Two cases from our studies suggest that empty niches are important in the persistence of polyploids. The recent origin of the high-montane forest and páramo habitats in Costa Rica is reflected in their depauperate floras relative to the world-level biodiversity to be found in the northern Andes. Empty niche space is likely to remain available in these habitats because of their distance from the source for new diversity in the northern Andes, so that our study allotetraploids *Polystichum lilianiae* and *P. talamancanum* have been more likely to persist there. Serpentine substrates where we found and developed our understanding of the allotetraploid *Adiantum viridimontanum* present substantial physiological challenges because of low calcium availability and high heavy-metal content. These features increase the empty niche availability already the case because of the small habitat-island size, distance from existing serpentine-adapted plants in maritime Canada and western North America, and recent (only 10,000 years) availability of the Northeastern serpentine outcrops.

In Hawaii, Wagner (1993) reports a high proportion of polyploids among the ferns, including high polyploids, similar to the pattern reported for Madeira by Manton (1950) and Lovis (1977). Similarly, in our Hawaii work on *Polystichum*, we encountered two polyploids, one an octoploid, but only one diploid. Wagner (1993) provided spore size measurements for the three Hawaiian *Polystichum* species we studied: using his length data to calculate an estimate of spore volume, tetraploid *P. haleakalense* is 1.25–1.5 times larger in volume than diploid *P. hillebrandii*, and octoploid *P. bonseyi* is between 3.8 and 4.8 times larger than the diploid. Spore size in *Adiantum*, documented by the work in our lab, shows the same size increase in the tetraploid. Further, the larger spore volume of *A. aleuticum* on eastern serpentine substrate, a series of Darwinian islands, is larger than its matrix-forest ally *A. pedatum*.

This pattern is interesting in relation to Lovis's 1977 contention that high polyploids are commoner on oceanic islands, because of the even greater increase in spore size and thus precinctiveness among these plants. High polyploids, increasingly more precinctive with the larger spores characteristic of higher ploidy levels, are more likely to persist on islands. In the Hawaiian archipelago, the octoploid is the commonest species (Wagner, 1993). Its distribution, unlike the other two, includes only a single site on the northwesternmost portion of the big island of Hawai'i, *i.e.,* nearest to the large population on adjacent Maui, the older island. This distribution is consistent with *P. bonseyi* only recently dispersing from the older island to the younger island, perhaps evidencing its increased precinctiveness in being less dispersible between islands than the other two species.

Origin, expansion, and persistence.—At a single origin, an allopolyploid has a narrowly restricted range and known progenitors; the polyploid and its progenitors are almost certainly sympatric. Hence, relatively young polyploids are more likely to have restricted ranges within the range of their extant progenitors. In our work, we have studied two different polyploid complexes where allotetraploids are narrowly restricted: North American *Adiantum* (one tetraploid) and Costa Rican *Polystichum* (two of the three tetraploids). Both the age of the lineages (Table 1) and the age of the habitats are recent, in line with recent origin. In these lineages, the tetraploids are sympatric with their progenitor diploids.

Two recently originated polyploids in our studies have broader ranges than their extant progenitors, with which they are still partially sympatric. Though the range of tetraploid *Polystichum californicum* has expanded beyond that of both of its progenitors, new sterile diploids (*i.e.,* candidate tetraploids) are still being produced. In the tropical Andes, we have revealed a chronologically young tetraploid with a broad distribution: the páramo tetraploid. The broad range of the young tetraploid includes two widely disjunct satellite populations, one where I first encountered this taxon in Sierra Talamanca and the second near the northern limit of the Neotropics at the border between Guatemala and Mexico. Since this tetraploid is younger than the other two *Polystichum* tetraploids in the Cerro de la Muerte, it is clear that the rate of expansion varies between polyploids, presumably due to a combination of stochastic and ecological factors (*e.g.,* the open-páramo species may disperse at distance more often; its spores are smaller than those of *P. talamancanum* [Barrington, Paris, and Ranker, 1986]), and forests do not impede the dispersal of its spores. In contrast, our study of *P. braunii*, an older polyploid with apparently extinct progenitors, suggests that the species occupied the current very broad range only recently. Expansion and displacement may be characteristic of polyploid lineages as posited by Manton and Stebbins, but there appears to be substantial variation in the pace and timing of geographic expansion of polyploid ranges relative to the range and historical fate of their progenitor lineages.

Two of the projects in our lab, both on *Polystichum* species, address polyploids whose diploid progenitors are not to be found and inferred age of the polyploids is earlier in the Neogene. The widespread tetraploid *Polystichum braunii*, in contrast to almost all other polyploids in eastern North America, seems best interpreted as originating from two extinct species that were sympatric in Beringia in the late Miocene—but likely extinct now. The austral South American *Polystichum* clade of seven polyploid species, nested in a clade with entirely polyploid species from New Zealand and Australia, presents the clearest case of a widespread lineage whose diploid antecedents are extinct. The Neogene ages of all these lineages preclude vicariance as the cause of the disjunct distribution, a conclusion drawn for many of the other lineages sharing this interrupted circumaustral distribution (Sanmartín and Ronquist, 2004). It seems likely that spores of an ancestral tetraploid lineage were dispersed over the long distances involved—but a clearer understanding of this history awaits genomic analysis of the polyploids across this whole austral lineage.

Polystichum is not the only genus with an entirely tetraploid lineage in Austral regions: a diverse array of *Asplenium* species in Australia and New Zealand also includes only polyploids (Shepherd, Perrie, and Brownsie, 2008). The Australasian *Asplenium* complex comprises both tetraploids and their descendant octoploids, thus presenting a pattern like that of austral South American *Polystichum* but more complex, better-resolved, and with more insight into its history. Preliminary dating of the *Asplenium* phylogeny (Shepherd, Perrie, and Brownsey, 2008) dates the origin of the polyploid clade to at least 10 mya, with a second burst of polyploid speciation among the tetraploids yielding octoploids in the Pleistocene. Together, the *Polystichum* and *Asplenium* patterns suggest that the southern continents harbor fern lineages comprising polyploids resulting from more ancient reticulation histories that have more recently become involved in a second wave of reticulation events. Thus, Austral regions harbor two old polyploid fern lineages that are diverse and widespread, while their progenitor diploids are almost certainly extinct—in concert with Stebbins's ideas. The longer histories may reflect the earlier onset of glaciation with its attendant disturbance in the southern hemisphere. These polyploids are not inherently doomed to extinction as Stebbins and Mayrose have predicted, at least in a Neogene timeframe.

There remains the case of Hawaiian *Polystichum bonseyi*, for which, based on our work, we have hypothesized an extinct tetraploid progenitor. Though it has an extinct progenitor, *Polystichum bonseyi* is neither old nor widespread. However, *P. bonseyi*'s insularity and large spore size argue for it being an acceptable exception to the idea of polyploids having expanded over time, followed by the decline and loss of their progenitors. Though successful migration away from the Hawaiian archipelago is argued to be possible (Heaney, 2007) the extraordinary isolation of the islands seems likely to have hindered range expansion for the octoploid. Further, the large spore volume of the octoploid increases precinctiveness and hence decreases dispersibility, adding to the challenge to range expansion. At the same time, the risk of extinction on Darwinian islands is greater (MacArthur and Wilson, 1967), so loss of progenitors earlier than in continental settings is likely, especially given the need for dispersal from older to younger islands of an archipelago.

CONCLUSIONS

The distribution of allopolyploids in the ferns in space and time is fundamentally different from the biogeography of divergent lineages because there is so little divergence yielding species among polyploid ferns. A sense of the biogeography of polyploid ferns comes from comparison of the ecology and geography of the origin, persistence, and extinction of individual polyploid species and their inferred or known progenitors. Modern work supports the origin of the polyploids being promoted by disturbance making hybridization more likely, just as Manton, Lovis, and Stebbins argued beginning eighty years ago. These origin events are more likely on Darwinian islands, that is, insular settings whether they be oceanic, edaphic, or montane (sky) islands. Thus, the biogeography of allopolyploid ferns is fundamentally grounded in island biogeography. The attributes of these islands that pertain to both the origin and success of allopolyploids are the greater levels of disturbance, the greater availability of open niche space, and the selection for larger propagule size, including larger spore size characteristic of polyploid ferns.

Polyploid ferns often expand and displace their progenitors over time, though the history of these expansions is diverse. It appears that polyploid ferns indeed persist longer on islands, where higher levels of ploidy are concentrated, perhaps because of the precinctiveness advantage that their larger spores provide these plants. However, this advantage may be limited by the challenge that inter-island dispersal presents to the dispersal of the larger spores, leading to higher extinction rates given the decline and loss of appropriate habitat on subsiding islands. The tendency for high polyploids on islands to lack extant progenitors may be a result of this higher extinction rate.

However, in general the polyploid ferns are not durable—they do not usually serve as the common ancestors of new polyploid lineages. Exceptions can be found in Austral regions, where two fern lineages have diverged at the tetraploid level while their diploid progenitors have all gone extinct. The geographic and climate feature shared by these southern regions that may provide insight into history is an earlier onset of glaciation and its incumbent high levels of divergence. How this history relates to the evidence for whole-genome duplication with polyploidy remains to be discovered.

Future Directions

Stebbins (1940), Manton (1950), and Lovis (1977) coined the ideas that are foundational to our understanding the biogeography of polyploids. What insights do we have that were not accessible to them? Our current understanding is informed by three kinds of information either completely unavailable to or not utilized by these authors: molecular sequence data, the phylogenetic inferences from these data captured in chronograms, and spore size. As a result, although most of the ideas are not new, the power to develop rigorous hypotheses about the biogeographic history of polyploid ferns has been improved immensely. Three future directions for research seem central. First, next-generation sequencing with a coalescence approach has the potential to provide a tool for identifying the progenitors of polyploids that can be used with herbarium specimens of substantial age. Second, significantly broadening the lineages for which we have high-quality dates-of-origin inferences for polyploids with fully resolved ancestries will improve our ability to test ideas about the origin and durability of polyploid species and lineages.

Finally, there is the essentially untapped potential to apply an orthodox vicariance-biogeography approach (Nelson and Platnick, 1981) to polyploid biogeography using comparisons of unrelated progenitor-descendant trios with similar geographic patterns. In this contribution, I have taken this approach informally in considering the two Austral fern lineages that lack diploid species—leading to my seeking geographic and climatic explanations common to the two. More formal approaches to multiple lineages in different geographic regions have the potential to improve our understanding of how climatic and geological factors influence the history of hybridization and polyploidy in the ferns.

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SPECIAL ISSUE

Celebrating David Barrington's Contributions to Fern and Lycophyte Research: Introduction to an American Fern Journal Special Issue
Weston L. Testo 145

Speciation and Reticulation in the *Polystichum* Allotetraploids of the Costa Rican Cordillera Talamanca
Stacy A. Jorgensen and David S. Barrington 151

Botrychium furculatum (Ophioglossaceae), a New Moonwort Species from the Rocky Mountains of North America
Steve J. Popovich, Donald R. Farrar, and Arthur V. Gilman 165

A New Species of *Goniopteris* (Thelypteridaceae) from Hispaniola
Susan Fawcett 183

There and Back Again: Reticulate Evolution in *Ceratopteris*
Sylvia P. Kinosian, William D. Pearse, and Paul G. Wolf 193

Diversity Patterns of Neotropical Ferns: Revisiting Tryon's Centers of Richness and Endemism
Jacob S. Suissa and Michael A. Sundue 211

The Biogeography of Polyploid Ferns Across Space and Time
David S. Barrington 233

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